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Award Number: DAMD17-99-1-9541

TITLE: Neuronal Degeneration in the Cingulate Gyrus: NMDA Antagonists and Anticholinesterases

PRINCIPAL INVESTIGATOR: Wilkie A. Wilson, Ph.D.
Suzanne Clark, Ph.D.
Qiang Li, M.D., Ph.D.
K. Jones, Ph.D.

CONTRACTING ORGANIZATION: Duke University Medical Center
Durham, North Carolina 27710

REPORT DATE: October 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2001	Annual (30 Sep 00 - 29 Sep 01)

4. TITLE AND SUBTITLE
Neuronal Degenration in the Cingulate Gyrus: NMDA Antagonists and Anticholinesterase

5. FUNDING NUMBERS
DAMD17-99-1-9541

6. AUTHOR(S)
Wilkie A. Wilson, Ph.D.
Suzanne Clark, Ph.D.
Qiang Li, M.D., Ph.D.
K. Jones, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Texas A&M University
College Station, Texas 77843-3000

E-Mail: ewing@vprmail.tamu.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Neurotoxic mechanisms and interactions between NMDA antagonists and acetylcholinesterase inhibitors (AChEIs)/cholingeric agonists were investigated using patch clamp recordings in rat brain slices of posterior cingulate/retrosplenial cortices (PCC/RSC), Functional Observational Battery monitoring, and Fluoro-Jade B (FJ-B) neurodegeneration staining. We obtained direct evidence that NMDA-mediated excitatory postsynaptic currents (EPSCs) in interneurons are blocked by NMDA antagonists (MK-801), the first direct evidence for their hypothesized neurotoxic mechanism. Physostigmine and pilocarpine suppressed GABAergic inhibitory postsynaptic currents; MK-801 was additive to them. In vivo, MK-801 is more neurotoxic in older than younger rats: in PCC/RSC slices, NMDA-mediated EPSCs were more rapidly suppressed by MK-801 in adults, suggesting a mechanism for age differences. Ethanol had more complex effects, and younger rats were more sensitive. In vivo, MK-801 alone produced FJ-B-positive neurodegeneration in the PCC/RSC, whereas, when used alone, AChEIs pyridostigmine and physostigmine did not. These findings are relevant to prevention of nerve agent exposures: NMDA antagonist drugs (e.g. for pain, neuroprotection, or recreation (e.g., ethanol)) could be co-administered with AChEIs (e.g., for soman prophylaxis). To avoid unexpected synergistic neurotoxicity in humans, the potential for serious neurotoxic interactions must be carefully explored in animals.

14. SUBJECT TERMS	15. NUMBER OF PAGES
neurotoxin	101
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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C. Functional Observational Battery (FOB) Behavioral Test in rats exposed in vivo to NMDAr/c antagonists and AChEIs.

INTRODUCTION:

The key objectives of these studies are to determine underlying mechanism by which NMDA antagonists produce region-specific neurotoxicity, and to determine the neurotoxic risks of combining agents that inhibit acetylcholinesterase (AChE) enzymes with agents that block N–methyl-D-aspartate receptors or channels (NMDA antagonists). There are three approaches by which we are attacking these objectives: patch-clamp electrophysiological recordings, behavioral assessment, and histopathological studies using Fluoro-Jade B.

BODY:

Background and Rationale

Recent events have returned to the forefront concerns that future terrorist attacks could involve chemical weapons – including the nerve warfare agents such as soman, sarin, or other acetylcholinesterase inhibitors (AChEIs). Given the difficulties encountered during the Persian Gulf War, it became clear that much work needed to be done to prepare for future attacks. Necessarily, these preparations must include identification of safe and effective measures to prevent toxicity (e.g., with prophylactic agents such as pyridostigmine bromide (PB)) or to treat exposed individuals (e.g., with anticonvulsants).

Accordingly, it is of fundamental importance to identify compounds that might impair the effectiveness of therapeutic drugs or, worse still, exacerbate the toxicity of nerve agents or the drugs used to combat their effects. One class of pharmacological

compounds that could exacerbate the toxicity of AChEI nerve agents (or related therapeutic agents) is the NMDA receptor/channel antagonists (NMDA antagonists). Evidence for this possibility was provided by an important study by Olney and colleagues (Corso, et al., 1997). These investigators found that pilocarpine, a muscarinic cholinergic agonist, greatly exacerbated the neurotoxicity of NMDA antagonists. However, it was not clear if AChEIs could also exacerbate this neurotoxicity by increasing the levels of the endogenous agonist acetylcholine. If so, this could have wide-ranging military and civilian repercussions, given the number of different of AChEIs to which both populations might be exposed. Add to that the wide range of available drugs that possess NMDA antagonist properties, and the potential scope of the problem increases, yet again.

Thus, it is important to determine if there are serious risks for co-administration of these groups of compounds – not only to identify interactions that enhance toxicity, but also so that therapeutically useful agents are not unnecessarily withheld from patients, for reasons based merely on theoretical toxic interactions that do not, in reality, exist. The need for such studies was directly addressed in a recent DOD News Briefing focused on USD Personnel and Readiness with respect to nerve gas attack (January 12, 2001) (Rostker, et al., 2001). Specifically, Dr. Ross Anthony (RAND Director) stated that, in reference to agents that might interact with PB or nerve agents: "... interactions could also [be] involved and need to be looked at".

To address these problems, we have taken a two-sided approach. In one group of experiments we have explored the mechanisms by which NMDA antagonists produce neurotoxicity – and the mechanisms by which cholinergic agents might exacerbate its

neurotoxicity – using patch-clamp electrophysiological recordings in rat brain slices. These studies are aimed at understanding the mechanisms of the neurotoxicity, with the hope that such understanding can lead to better prevention and treatment of future problems. Our second approach is focused on testing specific agents against one another, to determine which interactions worsen (or reduce) NMDA antagonist-induced neurotoxicity. These studies are aimed at identifying dangerous (and safe) AChEI and NMDA antagonist combinations in animal models, in order to improve therapeutic care of exposed military personnel and civilians exposed to nerve agents.

Issues relevant to the Persian Gulf War and future conflicts

The original impetus for this study was to understand the possible causes of the neurological symptoms of Persian Gulf War Syndromes (PGWSs) and related problems. During (and after) the Persian Gulf War (PGW), many difficulties were encountered with respect to prophylactic treatment against exposure to warfare nerve agent and other health-related issues. Ten years later, many problems and questions still plague those involved in that conflict. Individuals with confirmed or suspected diagnoses of one of the Persian Gulf War Syndromes (PGWSs) continue to search for answers, in hopes they can find causal conditions, proper diagnoses, and optimal treatments. The US Armed Forces and other government organizations also continue to search for answers, so that individuals suffering from the PGWSs can be better served, and so that in the future we can avoid unnecessarily exposing military personnel and civilians to conditions that cause serious health problems (Joseph, 1997; Haley, et al., 1997a,1997b, 2000; Riddle, et al., 2000, Sartin, 2000).

Suspected toxic agents in the PGW

A broad range of potential toxic agents have been identified to which PGW personnel could have been exposed, but no conclusive evidence has been found that would explain the symptoms from which the affected PGW veterans suffer (Riddle, et al., 2000; Rostker, et al., 2001). Some of the most worrisome agents to which PGW veterans may have been exposed are the anti-cholinesterase inhibitor (AChEI) warfare nerve agents. This includes individuals exposed during the post-war clean-up period, when Iraqi facilities containing nerve agents were demolished. Insect repellants and pesticides were also widely used. Based on a recent survey, Dr. Bernard Rostker, Under Secretary of Defense for Personnel and Readiness, and others reported that the insect repellent DEET was used by approximately 50 % of personnel in the Persian Gulf War, with the median frequency of use being about 30 times a month, depending on the service (Rostker, et al., 2001). This report identified other agents that were commonly used: approximately 6 % of the population used permethrin insecticides a frequency of about 20 times a month, 7 % used (or observed) fly strips in tents (sometimes up to 3 per space, and some of which contain organophosphates), 12 % used (or observed) fly bait and related products, and 3 % used (or misused) pet flea collars. Only one exposure to insecticides was sufficient to necessitate hospitalization, and that was a soldier exposed to leaking cans of insecticides in a warehouse. Most individuals who used insecticides did not report adverse effects. However, given the uncertainty of the effects of these doses and combinations, including co-exposure to PB or other risks, the role of insecticides cannot be completely ruled out.

Other agents have also been implicated, including suspected, but infrequent exposures to depleted uranium bombs and exploded armor, as well as mustard agents. However, results remain inconclusive and probably are not causal of any of the PGWSs. For example, a recent report by the DOD ("Reported Mustard Exposure: Operation Desert Storm") concluded that mustard exposure remained "indeterminate", so cannot be ruled out as causative (or contributory) agents in some of the reported illness (DoD News Release No. 309-01, 12 Jul., 2001). Still, the exposures to depleted uranium and mustard gases reflect a small percentage of the total number of individuals involved in the PGW.

However, one other group of compounds to which many individuals may have been exposed is the anti-nerve gas agents. Several of these agents have been proposed to be involved in chronic problems in PGW veterans, although no causal relationship has been established. This list includes pyridostigmine (PB), one of the drugs given to military personnel to prevent or attenuate poisoning due to AChEI-type nerve agents, especially soman, which undergoes rapid aging, making it very difficult to treat post-exposure (Golomb, 1999). Many individuals were given 30 mg PB tablets during the PGW, and although the exact number and specific individuals exposed has been difficult to determine, "a great majority" may have taken at least some tablets, and some individuals may have taking many more of the tablets (or taken for longer periods) than advised (Golomb, 1999). Accordingly, it is on this agent that much emphasis has been focused.

Moreover, this is not an issue that is confined only to concerns regarding the PGWSs. The future use of PB should be expected, given the dangers of warfare nerve

agents, the effectiveness of PB in animal models to reduce incapacitation and mortality due to exposures to AChEI warfare agents, and the relative safety of PB when used clinically. This expectation was outlined in a statement (*The Use of the Drug Pyridostigmine Bromide as it relates to Gulf War Veterans*) submitted on Nov. 16th, 1999 to the Subcommittees on Health and Oversight and Investigations Committee on Veterans Affairs by Dr. Sue Bailey, the Assistant Secretary of Defense for Health Affairs. In the opinion of the author, at that time of writing, the multiple studies and reviews on PB did not provide definitive evidence that PB should be withheld from use as a pre-treatment drug (assuming that it is used in conjunction with standard post-exposure treatments) if there is a "confirmed high threat of the use of soman". The use against soman is highlighted, because soman is rapidly active, the "standard treatments for other nerve agents are not effective against soman". Also, it is suspected that soman is in the arsenals of some groups opposed to the US.

Indeed, given the potentially fatal outcome of soman or sarin exposure, it would be inexcusable not to use PB during the threat of an attack, unless better alternatives exist. However, until a better agent is developed, it is important to identify risk factors that might increase its toxicity, because PB may not be well tolerated in all people or under all conditions. For example, during the Persian Gulf War, some military personnel taking PB reported symptoms that suggested excess cholinergic activity, although these symptoms were reversible when the drug was discontinued (Caldwell, J.A., 1992). At the time, the reversibility of the symptoms suggested that no long-term problems would exist with the use of PB. However, subsequent problems (the PGWSs) have required a re-

evaluation of the effects and interactions that may occur in the event of prolonged use of this agent.

Factors proposed to increase PB toxicity:

Several factors have been reported to increase the neurotoxicity of PB in vivo, and some may have played a role in the acute – and subsequent chronic, problems in PGW personnel. For example, while PB does not normally cross the blood brain barrier, acute stress may produce conditions that allow PB access to the CNS (Friedman, et al., 1996). Also, endogenous enzyme levels may influence toxicity, for some individuals (and animal strains) possess reduced levels of enzymes that scavenge PB, a condition that may increase vulnerability to the toxicity of AChEIs. For example, in rats exposed for 7 days to PB in their drinking water, the acoustic startle response was impaired in a rat strain (WKY) with reduced butyrylcholinesterase (BuChE) activity compared to the Sprague-Dawley (SD) rat strain (Servatius, et al., 1998).

In addition, the duration of exposure may influence neurotoxicity: studies in animals suggest that chronic exposure to PB may have different long-term effects compared to acute exposures. Rats given PB (0.5 to 1.85 mg/kg) twice daily for 4 days had signs of neuronal degeneration in the cerebral cortex, striatum and hippocampus: apoptotic neurons were found using TUNEL staining, *In Situ* Cell Death Detection methods, or electron micrographic techniques (Li, et al., 2000). Although these effects might be explained by toxicity arising secondarily to peripheral stress induced by PB, in vitro studies by these investigators suggested that PB (250 μM) was directly toxic to cultured cortical neurons (Li, et al., 2000). Thus, if certain conditions can alter the blood

brain barrier to allow PB to cross, it might be directly toxic to neurons, if sufficiently high levels are reached. Furthermore, PB-induced neurotoxicity can be exacerbated by other drugs. Studies in mice (Chaney, et al., 1999) and chickens (Abou-Donia, et al., 1996) suggest that, when PB is used in combination with other drugs and chemicals (e.g., the insect repellant DEET), unexpected neurotoxic effects may develop. Moreover, DEET and other previously studied drugs may not be the only agents that might interact negatively with AChEI's. There is good reason to suspect that other classes of drugs might pose similar problems, including, as discussed below, NMDA antagonists.

NMDA receptor/channel antagonists ("NMDA antagonists")

When first developed, the NMDA receptor/channel antagonists ("NMDA antagonists") were hailed as a potential class of wonder drugs, holding promise for a range of medical conditions, including as neuroprotective agents and anticonvulsants. However, clinical trials did not always prove to be successful, and in some cases the drugs caused severe psychotic reactions (e.g., see Sveinbjornsdottir, et al., 1993). Some more recently developed agents have proved to be effective in certain clinical conditions, as well as more tolerable, and suggest that, at least some types of NMDA antagonists may hold clinical promise. However, the concern about neurotoxic side effects persisted, and until the mechanism was determined, this concern would limit the use of these agents.

The mechanism remained a mystery until Olney and colleagues discovered that NMDA antagonist produced neurodegeneration in certain areas of the brain (Olney et al., 1989 & 1991). Thus, the fears that NMDA antagonists could produce long-lasting

problems were realized. However, now that the anatomical targets were known, hopes were raised that non-toxic agents could be developed.

Clinical and military relevance regarding NMDA antagonists, AChEIs and other agents:

There are a broad range of drugs and investigational agents that have been found to possess NMDA receptor/channel antagonist activity. For some drugs, this characteristic may be a major mechanism of action responsible for the therapeutic action of a particular drug or agent (e.g., the anesthetic agent ketamine or the research tool dizocilpine (MK-801)). For other drugs, NMDA receptor antagonist activity could be one of several co-existing therapeutic mechanisms, one or more of which may contribute to the clinical effectiveness of that drug (e.g. the anti-Parkinson's agent memantine (Tomitaka, et. al, 1996) or the anticonvulsant felbamate (Rho, Donevan & Rogowski, 1994).

Anticonvulsants could have significant relevance during warfare nerve agent attacks, because anticonvulsants are administered to treat the serious seizures that can arise after a high-level exposure. The importance of treating soman-induced convulsions was highlighted in a recent study. It was found that soman-exposed rats that had exhibited soman-induced convulsions were likely to have hippocampal damage (in area CA1) and memory impairment, whereas rats exposed to an identical dose of soman, but in which convulsions did not occur, were spared (Filliat, et al., 1999). Furthermore, drugs with mixed actions as anticholinergics and NMDA antagonists may hold special promise for use against nerve agent-induced seizures (McDonough & Shih, 1995), perhaps due in part to the stages through which nerve agent-induced seizures progress, as

indicated by a sequential sensitivity to anticholinergics in the early stages, a transition phase, and a late phase in which anticholinergic agents are no longer effective (whereas benzodiazepines and NMDA antagonists are effective if co-administered with anticholinergics) (McDonough & Shih, 1997). These findings have important clinical implications, for effective treatment during the early phase of seizures prevented neuropathology, but when treatment was delayed until the late phase was reached, neuropathology occurred.

Although most commonly used anticonvulsants are not thought to possess significant NMDA antagonist activity (e.g., diphenylhydantoin or carbamazepine), several newly developed anticonvulsants may possess sufficient activity at NMDA receptors to contribute to the therapeutic action. As mentioned above, one such anticonvulsant is felbamate, a recently developed and highly effective anticonvulsant. Although felbamate is not widely used because of serious hepatic and hematological adverse effects, it was such an effective anticonvulsant that similar, less toxic, agents will no doubt be developed in the future. Accordingly, it can be expected that some of those new drugs will possess a similar pharmacological profile, including activity as NMDA receptor antagonist.

Such specifically developed anticonvulsants might be important for treating seizures caused by AChEI nerve gas exposures, given that the NMDA antagonist MK-801 provided protection against soman-induced seizures in vivo (Braitman & Sparenborg, 1989). Furthermore, in studies using mice, high doses of pyridostigmine bromide (PB) and N, N-diethyl-m-toluamide (DEET)) cause seizures that were relatively

resistant to standard anticonvulsants (Chaney, et al., 1999). Thus, novel anticonvulsants – including those with NMDA antagonist activity – might be necessary for treating seizure that arise from unexpected toxicities associated with high doses of these drugs (e.g., some PGW personnel were reported to have taken more PB tablets than advised (Golumb, et al., 1999)) - or combinations of drugs that exacerbate the toxicity of each other (e.g., DEET and PB).

However, there is evidence that anticonvulsants with NMDA antagonist activity might not be effective against certain types of seizures. For example, dextrorphan is an NMDA antagonist related to a common antitussive with neuroprotective actions in some studies (Britton, et al., 1997). However, seizures induced by the combination of PB+DEET may have been exacerbated by the NMDA antagonist dextrorphan (25 mg/kg). Specifically, when dextrorphan pretreatment was tested against PB (3 mg/kg) +DEET (200 mg/kg)-induced seizures and lethality in mice, the results suggested that dextrorphan actually might *worsen* both the incidence of seizures and lethality compared to control mice or mice pre-treated with a traditional anticonvulsant (diazepam, fosphenytoin, or phenobarbital) (Chaney, et al., 1999). Although this effect of dextrorphan was not statistically significant, it is interesting that it was the *only* agent that produced a change in the direction of worsening the outcome, whereas the traditional anticonvulsants produced either no change or decreased seizures or lethality. In another study, soman-induced lethality was *increased* in rats given the non-competitive NMDA receptor antagonist N-[1-(-2-thienyl)cyclohexyl]-piperidine (TCP) and the AMPA antagonist 2,3-dihydroxy-6-nitro-7-methyl-4-sulphamoylbenzo-quinoxaline (NBQX) where, in contrast, atropine decreased the lethality of soman (FIlliat, et al., 1999).

Other agents:

Furthermore, certain drugs of abuse (e.g., phencyclidine (PCP)), legal drugs diverted for abused (e.g., the anesthetic ketamine), or legal recreational drugs (e.g., ethanol) also possess NMDA antagonist activity. These drugs may have inherent neurotoxicity, or may interact with other drugs to synergistically produce neurotoxicity. For example, chronic use of PCP in humans produces persistent psychotic symptoms that may mimic a chemical model of schizophrenia (Jentsch & Roth, 1999), and antipsychotic drugs also block NMDA antagonist-induced neurotoxicity (Farber, et al., 1996; Tomitaka, et al., 2000). In humans acutely exposure to ketamine, blood flow increases in the frontal lobes, especially in the cingulate and frontomedial regions (Jentsch & Roth, 1999). Because of its wide use, ethanol may provide the most worrisome opportunity for neurotoxic interactions. Ethanol is a non-competitive NMDA antagonist (Lovinger, White, and Weight, 1989; Chu, et.al, 1995). This property may have functional expression in humans. For example, recently detoxified alcoholics reported that ketamine produced ethanol-like subjective effects in double-blinded studies, suggesting that ethanol may be producing some of its intoxicating effects by antagonizing NMDA receptor function (Krystal, et al. 1998). These results suggest that ethanol concentrations reached in humans during intoxication are within a range that may affect NMDA receptor function. And ethanol may interact and potentiate other NMDA receptor antagonists: Corso, et al. (1992) reported preliminary evidence that ethanol given at non-toxic levels (100 mg %) potentiated MK-801 neurodegeneration. During fetal development, ethanol can cause massive neuronal loss through an action as an NMDA antagonist, but perhaps through a mechanism different than what occurs in adults (Ikonomidou, et al., 2000). The

neurotoxicity of repeated high doses of ethanol alone has been explored in more detail by Collins, Corso, and Neafsey (1996), however, neurotoxic interaction between ethanol and MK-801 has not.

Thus, as more drugs are being developed that are NMDA antagonists, such combinations are likely to occur therapeutically in military or civilian settings. For example, these agents may be used in combination as preventive measures to reduce toxicity from chemical warfare that act on AChE enzymes (e.g., soman), or to treat symptoms of exposure to chemical warfare agents, or may result from environmental exposures (e.g., organophospate insecticides).

In summary, the conditions of military service are dangerous enough, without adding to these dangers by administering treatments with serious, but unidentified, neurotoxic interactions. The same is true for civilians exposed to terrorist attacks – which, unfortunately, are now more likely than previously supposed. We hope that these studies will advance the knowledge about potential risks, and to help provide the best possible medical care for those who serve us during dangerous times.

I. ELECTROPHYSIOLOGICAL STUDIES:

A. Overview of Electrophysiological Studies

B. MK-801, an NMDA Receptor Antagonist, Modulates the Inhibitory Postsynaptic Currents (IPSCs) in Pyramidal Neurons in the Rat Cingulate Gyrus (discussion of accepted manuscript included in Appendicies)

C. Excitatory drive onto PCC/RSC interneurons is reduced by NMDA antagonists: direct evidence for proposed mechanism of NMDA antagonist-induced neurotoxicity.

D. Cholinergic agonist and AChEIs may exacerbate NMDA antagonist-induced neurotoxicity through related mechanisms.

E. Differential effects of ethanol on NMDA receptor-mediated EPSCs in pyramidal cells in the posterior cingulate cortex of juvenile and adult rats (discussion of accepted manuscript included in Appendicies)

F. Influence of animal age on NMDA antagonist-induced neurotoxicity in the PCC/RSC: possible neurotoxic mechanisms.

I. A. Overview of Electrophysiological Studies

Completed studies: In the first two years of this project, we have employed whole-cell patch clamp recordings of individual neurons in rat brain slices to test hypotheses put forth regarding the mechanisms by which NMDA antagonist produce neurotoxicity in specific areas of the brain. In the first year of the study, we obtained recordings of $GABA_A$-mediated currents in pyramidal cells, and tested the effect on these responses of a range of concentrations of (5R,10S)-(+)-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801), as described in the previous Annual Report.

To more directly test these hypotheses, in the second year of this study we have recorded responses in interneurons in relevant areas in the PCC/RSC. Specifically,

interneurons were identified visually and by their characteristic firing patterns using patch-clamp recordings, and then NMDA receptor-mediated EPSCs were isolated and used to assess the effect of MK-801.

We have also explored additional factors that influence this neurotoxicity, including cholinergic inputs, exposure to other agents (e.g., ethanol), and animal age. Specifically, we have applied the cholinergic agonist pilocarpine in a range of doses and measured its effect on responses recorded in the PCC/RSC. Next, pilocarpine and MK-801 were applied together to assess the extent to which they interact. These experiments were repeated using a range of doses of the AChEI physostigmine. In addition, the competitive NMDA antagonist APV had a similar effect to MK-801 on IPSCs in pyramidal cells of the PCC/RSC. This is consistent with reports that competitive (e.g., CPP), as well as uncompetitive (e.g., MK-801) NMDA antagonists can cause neurodegeneration in the PCC/RSC (Hargreaves, et al., 1994; Olney, et al., 1991).

Detailed descriptions of these experiments are discussed in the attached manuscripts and abstracts (see appendix) or are outlined below (See topics I.B. – I.F.).

Reviewers' comments: We have acted upon the suggestions and comments provided by the reviewers of our First Year Annual Report; these actions will be discussed in the relevant sections outlined below.

Future work: In the past two years we have completed the majority of the intracellular electrophysiological experiments outlined in our original proposal.

Remaining experiments outlined in the original proposal using patch-clamp techniques are as follows:

(1) Record from interneurons in the parietal cortex, and compare the effect of MK-801 on the NMDA receptor-mediated EPSCs with the results obtained in the PCC/RSC.

(2) Complete the ongoing studies using pilocarpine and physostigmine.

(3) Complete the section "Electrophysiological analyses of non-prototypical compounds". This will involve finishing the ongoing studies using ethanol, and then using the three other drugs (memantine, felbamate, and dextromethorphan) which are clinically used and which possess some degree of NMDA receptor antagonist activity.

(4) Finally, we will complete the section "Electrophysiological analysis of brain slices from treated animals" using slices from rats previously exposed to a dose of MK-801. Based on our previous experience in our in vivo model, a dose will be chosen that reliably produces neurotoxicity in the PCC/RSC, but not parietal cortex. As outlined in the proposal, tests will be done to assess the neurophysiological responses in anatomical areas (and, within those areas, specific cell layers) that we have previously determined produce neurotoxicity (based on Fluoro-Jade studies).

I. B. MK-801, an NMDA Receptor Antagonist, Modulates the Inhibitory Postsynaptic Currents (IPSCs) in Pyramidal Neurons in the Rat Cingulate Gyrus

(This is a brief discussion of the manuscript included in Appendices.)

Review of first year work and finding obtained from the electrophysiological studies

In the first year of this project, we used rat brain slices that included posterior cingulate cortex and retrosplenial cortex (PCC/RSC) and/or parietal cortex and compared the effect of a prototypical NMDA antagonist (MK-801) on inhibitory input onto pyramidal cells in these different brain areas. These experiments were designed to explore the region-specific neurotoxic mechanism of NMDA antagonists. Olney had hypothesized that the mechanism by which NMDA antagonists produce neurotoxicity might be via region-specific disinhibition. Our initial findings provided evidence in support of this hypothesis. Specifically, we measured bicuculline-sensitive GABAergic inhibitory input onto pyramidal neurons, and compared the effect of MK-801 in an area highly vulnerable to NMDA antagonist-induced neurotoxicity (the PCC/RSC) to the effect in a more resistant area (the parietal cortex). We hypothesized that MK-801 would disrupt bicuculline-sensitive GABAergic inhibition to a greater extent in the PCC/RSC than in the parietal cortex. Our results supported this specific hypothesis and, accordingly, Olney's more general hypothesis. In addition, these initial have suggested new avenues of study with respect to this project.

Completion of initial findings:

Since the first year's study, additional experiments were performed to expand upon the above-mentioned initial findings and to address specific issues important to this study (see appendices).

The completed manuscript was submitted to The Journal of Neuroscience. The manuscript was enthusiastically and thoughtfully reviewed and has been accepted for publication, pending revisions. The revisions were based on the reviewers' reasonable requests for changes in the text and additional experiments (see attached reviewers' comments).

All of the additional experiments requested by the reviewers have been done, and the written revisions are in the final stages. (Please note that the attached manuscript reflects the version originally sent to The Journal of Neuroscience. That is, it is the version *before* the final revisions and additional experiments were completed. The final version will be provided once it is in completed form.)

Overall, the reviewers noted the importance of the topic of this study, and their comments and suggestions have further strengthened the paper. Furthermore, the results of the additional experiments have supported our original findings.

The other patch-clamp electrophysiological experiments performed in this second year have taken the initial results to a new level and have addressed many of the questions put forth in our initial proposal. Accordingly, we will discuss the experiments and results in the order in which the experiments were proposed in our original proposal, starting with recordings from interneurons.

Materials and Methods for Second-Year Studies

(The following general methods apply to the following, and subsequent studies in this section describing electrophysiological studies done in the second year of this project.)

Cortical slices

Cortical slices were prepared from young (postnatal day (P) 15-25) male Sprague-Dawley rats. In experiments focused on the influence of animal age, animals from the following age groups were used: young (P 10-20), juvenile (P30-35) and adult (P130-140) male Sprague-Dawley rats. Rats were iosflurane-anesthetized and decapitated. The brains were quickly removed and placed in cold (4°C) artificial cerebrospinal fluid (aCSF) saturated with 95%O_2/5%CO_2 and containing (in mM) 120 NaCl, 3.3 KCl, 1.23 NaH_2PO_4, 25 $NaHCO_3$, 1.2 $MgSO_4$, 1.8 $CaCl_2$ and 10 D-glucose at pH 7.3. Coronal cortical slices containing the PCC/RSC or the parietal cortex (Paxinos and Watson, 1986) (300μm thickness) were cut with a vibratome (Campden, Model 752, England) and incubated in a holding chamber continuously bubbled with 95% O_2/5% CO_2 at room temperature (22-24°C).

Whole cell voltage-clamp recording

For recording, patch pipettes were pulled from borosillicate glass capillary tubing (1.5mm O.D., 1.05 mm I.D., World Precision Instrument, Sarasota, FL) on a Flaming-Brown horizontal microelectrode puller (Model P-97, Sutter Instrument Co, Novato, CA). Pipettes were filled with an intracellular solution containing (in mM) 130 Cs-gluconate, 7 CsCl, 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEEPS), 4 Mg-ATP (pH=7.25). The quaternary lidocaine derivative QX-314 (4mM) (Sigma Chemical Co., St

Louis, MO) was also included to suppress fast sodium currents. Osmolarity was adjusted to 280 mOsm. Pipette resistances generally were in the range of 4-7MΩ. Biocytin (0.3~ 0.4%) (Sigma Chemical Co., St Louis, MO) was also added to the intracellular solution for later visualization of the morphology of the recorded cells.

In order to obtain the firing pattern of cortical interneurons, the tip of the patch pipette was filled with the K-gluconate internal solution containing the following composition (in M): 130 K-gluconate, 10 KCL, 10 HEPES, 4 Mg-ATP, and 0.4GTP (pH 7.25, 285 mosM) and backfilled with a solution containing Cs+ (see above). The dialysis of the recorded cell with the Cs-gluconate solution could be observed from the broadening of the action potentials after ~10 min of recordings.

After > 1 hour of incubation in the holding chamber, a slice was transferred to a small submersion chamber maintained at room temperature (22-24°C) and secured in place with a bent piece of platinum wire resting on the top of the slice. Individual cells were visualized using an infrared differential interference contrast (IR-DIC) Zeiss Axioskop microscope and a 40X water immersion objective. Tight seals (>1GΩ.) were obtained on pyramidal-shaped cells and whole cell recordings were made after rupturing the cell membrane with gentle suction. After establishment of the whole-cell recording configuration, stable long lasting tight-seal recordings were achieved in most cases. Spontaneous and evoked IPSCs were recorded continuously using an Axopatch 1-D amplifier (Axon Instrument Inc, Foster City, CA). Output current signals were DC-coupled to a digital oscilloscope (Nicolet Model 410). Series resistance was monitored

throughout the recordings; a cell was discarded if it changed significantly. In addition, a PCM/VCR recorder (Model 400, A.R. Vetter Co, Rebersburg, PA) was used to capture all tracings of synaptic events for off-line analysis and archiving. The stored signal was further analyzed using Strathclyde Electrophysiology Software Whole Cell Program (Courtesy of Dr. John Dempster) with an interface (BNC-2090, National Instruments, Austin, TX) to a PC-based computer.

Electrical stimulation

A monopolar tungsten electrode (A-M system, Inc, Carlsborg, WA) was placed about 50~70μm lateral to the soma of the recorded pyramidal cells in the same layer. The stimulus threshold was first determined by increasing the intensity of the rectangular wave pulse until detectable responses occurred. Then constant current rectangular stimulus pulses 50% higher than threshold intensity with the duration of 0.1ms and frequency of 0.0166Hz were delivered through the electrode by an isolated stimulator (Grass S88, Grass Instrument CO, Quincy, MA).

Histological identification of pyramidal cells.

During recording, pyramidal cells were filled with biocytin. After the end of the recording the slice was held in the recording chamber for an additional 10-20 minutes to allow for biocytin transport along the axon. The slices were then placed overnight in 4% paraformaldehyde and 0.05% gultaraldehyde in 0.1M phosphate buffer saline (PBS). The slices were washed thoroughly in PBS and were incubated in 0.1 M Tris-buffered saline (TBS) containing 1% H_2O_2 for 30 minutes. The slices were then incubated with avidin-biotin-peroxidase complex (ABC kit, Vector Labs, Burlingame, CA) in TBS containing

0.05% Triton X-100 overnight at 4 ℃. The slices were then rinsed 3 times in PBS, reacted in a solution containing DAB (DAB kit, Vector Labs, Burlingame, CA), then cleared and mounted. The morphology of the biocytin-filled pyramidal cells was examined with a light microscope and pyramidal cells were drawn using a camera lucida.

Statistical analysis of data and drug application

Data were analyzed off-line using Strathclyde Electrophysiological Software. Paired and unpaired *t*-tests and one-way ANOVA tests were also used, when appropriate. All group data are presented as mean ± SEM.

MK-801 (dizocilpine maleate) and tetrodotoxin (TTX) were purchased from RBI (Research Biomedical International, Natick, MA). D-(-)2-amino-5-phosphonovaleric acid (D-AP5), 6,7-dinitroquinoxaline-2,3-dione (DNQX) and bicuculline methiodine (BMI) were purchased from Sigma (Sigma, St, Louis, MO). All drugs were dissolved directly in the aCSF and bath-applied in the perfusion medium.

I. C. Excitatory drive onto PCC/RSC interneurons is reduced by NMDA antagonists: direct evidence for proposed mechanism of NMDA antagonist-induced neurotoxicity

Introduction: recording from interneurons in the PCC/RSC

In our original application, we proposed to "record from cells that are identified as interneurons by their appearance and firing pattern". The rationale for looking at interneurons was that this approach would provide the most direct test of Olney's general hypothesis regarding NMDA antagonist-induced neurotoxicity. Specifically, if inhibitory interneurons receive excitatory drive via NMDA receptors, then NMDA antagonists could disrupt this drive, leading to functional disinhibition. If sufficient, such disinhibition could leave that area of the brain vulnerable to hyerexcitability and excitotoxicity.

In our previous year's work, we showed that by monitoring activity of IPSCs in PCC/RSC pyramidal cells, inhibitory input to the principal cells can be reduced by bath application of MK-801 (40uM). These data, therefore, indirectly support the Olney's hypothesis. We have since then recorded from inhibitory interneurons in the PCC/RSC, have identified the interneurons by their appearance and firing pattern.

Results

It is generally difficult in making recordings from interneurons, since they are few in number, the soma are small in size, and are difficult to identify visually under RI-DIC (Figure-1A). However, interneurons fire rapidly upon depolarization and do not have frequency adaptation (Figure –1B). We took advantage of this characteristic by using a recording pipette filled with a solution containing potassium at its tip, and then backfilled

with a Cs+ containing solution (see Methods). The latter usually providing a tight seal for whole cell configuration for long-lasting recording. In so doing, we can first examine the firing patterns of the presumed interneuron to determine if the cell actually is an interneuron, and then monitor the cell continuously during the rest of the experiment. In addition, the morphology of the recorded biocytin-filled cell subsequently will be examined to confirm the identification of the cell type and location (i.e., the cortical layer in which the soma is located).

Thus, these methods allowed us to *directly* recorded spontaneous NMDA receptor mediated EPSCs in PCC/RSC interneurons, and to examined the effect of MK-801 on these NMDA EPSCs.

Figure-1 shows an example of an interneuron recorded in PCC/RSC layer III. In the presence of the GABA-A receptor blocker BMI (20uM) and at holding potential of –70mV, fast inward currents recorded from this interneuron are recorded. These fast inward currents can be completely blocked by the AMPA receptor blocker DNQX (20uM), indicating that they are mediated by AMPA receptors. There are no NMDA receptor-mediated inward currents at this holding potential, due to their voltage dependence. When the cell was held at +50mV, slow outward currents were observed (Figure-1D). These slow outward currents can be blocked by NMDA receptor blocker D-AP5 (50uM), indicating that these slow outward currents are mediated by NMDA receptors (data not shown). 20minutes after bath application of MK-801 40uM, there are no slow outward currents (Figure-1E). So far, we have isolated NMDA receptor mediated EPSCs from a total of 4 RSC III interneurons and found that MK-801 blocks all NMDA receptor-mediated EPSCs These findings suggest that interneurons in the PCC/RSC area

receive glutamatergic excitatory input, and that NMDA receptor mediated inputs to the interneurons can be blocked by non-competitive blocker MK-801.

Future studies:

In future studies we will increase the number of interneurons from which we record in the PCC/RSC and then record from interneurons in the parietal cortex. These recordings will be compared to determine if the difference between PCC/RSC and parietal cortex that was seen previously in IPSCs is extended to the difference between excitatory inputs onto interneurons.

I. D. Electrophysiological Studies: Cholinergic Agonist and AChEIs May Exacerbate NMDA Antagonist-Induced Neurotoxicity Through Related Mechanisms

Introduction:

As discussed above, Olney and colleagues reported that cholinergic agonists exacerbated the neurotoxicity in the PCC/RSC due to NMDA receptor antagonists (Corso et al., 1997) and that muscarinic cholinergic antagonists could protect against this toxicity (Olney, et al., 1991). Thus, cholinergic inputs may pay a role in modulating the neurotoxicity. The exact mechanism by which this could occur is not known, but it could be a network effect (that is, NMDA antagonists may have an effect on the cellular network. For example, NMDA antagonists could be acting distally on the cell bodies of cholinergic neurons (or neurons that influence their firing) in their nuclei. This could have a net effect of increased firing at cholinergic synapses in the PCC/RSC and other vulnerable areas. NMDA antagonists could also have a more local effect on the PCC/RSC neurons or network.

Either way, the net outcome suggests that there could be risks in co-exposures to cholinergic agonists and NMDA antagonists. Although cholinergic agonists represent only a small number of therapeutic agents used clinically, a much larger group of agents to which the military and civilian populations might be exposed are the AChEI's. This includes organophosphate and carbamate insecticides, as well as AChEI warfare nerve agents (e.g., soman and sarin) and therapeutic agents aimed at preventing the toxicity of these agents (e.g., PB), as well as cognitive enhancing drugs used in Alzheimer's (Taylor, 1998). However, no extensive studies have been done to identify the extent to which these agents might interact, or the possible mechanisms of action of this interaction. However, as discussed above, several results have been reported that suggest that some NMDA antagonists might exacerbate the toxicity of some AChEIs (e.g., dextrorphan versus PB+DEET (Chaney, et al., 1999) and non-toxic levels of ethanol (100 mg %) and MK-801 (Corso, et al., 1992).

Accordingly, we have tested the effect two cholinergic agents against IPSCs recorded in pyramidal cells in the PCC/RSC. We then compared the effect of these agents to the effect of MK-801, including co-applications of both an AChEI and MK-801 to determine if the agents occlude the effect of the other, or if the effects might be additive. The muscarinic agonist pilocarpine was chosen, since it is known to exacerbate NMDA antagonist neurotoxicity (Corso, et al., 1997). physostigmine was chosen as a representative AChEI that can cross the blood-brain barrier, and that can protect against AChEI warfare agents.

Results

Pilocarpine +/- MK-801:

We measured the responses of evoked IPSCs of PCC/RSC pyramidal cells in layer II and III to bath application of pilocarpine (2, 5 and 10uM) and to the combination of pilocarpine (10uM) and MK-801 (40uM). The evoked IPSCs were isolated from PCC/RSC pyramidal cells whose membrane potentials were voltage clamped at +5mV.

Figure-2A shows the response of evoked IPSCs of a PCC/RSC layer III pyramidal cell to pilocarpine. Sequential concentrations of pilocarpine were bath-applied at concentrations of 2, 5 and 10μM for 15 minutes each, and peak amplitudes of evoked IPSCs were decreased by 14.9, 31.3 and 34.5%, relative control responses, respectively. When MK-801 (40μM) was added into the bath solution containing pilocarpine (10μM), the peak amplitude of the evoked IPSCs was further decreased by 49.4% to the control.

We have tested the effect of pilocarpine on peak amplitude of evoked IPSCs on 8 PCC/RSC pyramidal cells. Of these 8 neurons, the combination of pilocarpine (10 μM) and MK-801 (40μM) was used on three cells. Figure-3B was a bar graph showing the effect of pilocarpine and MK-801 on the peak amplitude of evoked IPSCs. On average, pilocarpine at concentrations of 2, 5 and 10 μM reduced the mean peak amplitude of evoked IPSCs by 12.7 (n=8), 29.9 (n=8) and 41.2 (n=8) relative to the control, respectively. When pilocarpine (10μM) and MK-801 (40μM) were co-applied, mean peak amplitude of evoked IPSCS was decreased by 47.7% (n=3) relative to the control. One way ANOVA shows there is significant different changes in peak amplitude of

evoked IPSCs after bath application of pilocarpine and MK-801 ($F_{[3]}$=12.3285, P=0.00518).

Physostigmine +/- MK-801:

In these experiments, we have examined the effect of bath-applied physostigmine on the peak amplitude of evoked IPSCs recorded from PCC/RSC pyramidal cells. A range of doses (2-100 μM) were used that encompass effective doses used in previous in vitro studies (Zhang, et al., 1997). As shown in Figure-3A, physostigmine reduced peak amplitude of evoked IPSCs recording in a dose-dependent manner. Physostigmine reduced peak IPSC amplitudes by 11.9% (2 μM), 16.3% (5μM), and 24.9% (10 μM) compared to aCSF control. The maximal decrease (45.4%) in peak amplitude of evoked IPSCs was obtained when 100uM physostigmine was applied.

As shown in Figure 3B, similar dose-dependent results were obtained in a different pyramidal cell, and when MK-801 (40 μM) was added to physostigmine (100 μM), a profound reduction in IPSC amplitude occurred.

We have tested the effect of physostigmine on peak amplitude of evoked IPSCs on 6 PCC/RSC pyramidal cells. Of these 6 neurons, the combination of physostigmine (100μM) and MK-801 (40μM) was used in 3 cells. The bar graph in Figure-3C shows the inhibitory effect of physostigmine and MK-801 on peak amplitude of evoked IPSCs. On average 2, 5, 10 and 100 μM physostigmine reduce mean peak amplitude of evoked IPSCs by 10.3 (n=6), 18.2 (n=6), 32.1 (n=6) and 44.1% (n=3) relative to the control, respectively. When physostigmine (100μM) and MK-801 (40μM) were co-applied, the mean peak amplitude of evoked IPSCS was decreased by 50.3% (n=3), relative to the control. A one way ANOVA shows there is a significant difference in peak amplitude of

evoked IPSCs after bath application of pilocarpine and MK-801 ($F_{[4]}$=9.76403, P=0.00182).

Discussion and future studies:

These results suggest that cholinergic mechanisms can modulate inhibitory input onto PCC/RSC pyramidal cells, and may do so in a way that is additive with the effect of NMDA antagonists. This could be the underlying mechanism by which these two classes of agents interact, such that NMDA antagonist-induced neurotoxicity is exacerbated. Further experiments will need to be done to determine if a higher dose of either pilocarpine or physostigmine can occlude the effect of MK-801 (thus suggesting, although not definitive proof, that these two agents work by shared mechanisms). However, care must be taken to assure that toxic doses are not given, based on the report that high concentrations of pyridostigmine (250 μM) induced apoptosis in cultured cortical cells after a 24-hour exposure (Li, et al., 2000). If either pilocarpine or physostigmine reach a plateau at high doses (without causing toxicity), and if still MK-801 can cause further inhibition above that plateau, then perhaps the two agents work through different, additive mechanism, which might further explain their synergistic activity. Indeed, it is curious that in our in vivo studies, we have never seen histological signs of neuronal degeneration with either physostigmine or PB, suggesting that the effect of these agents alone is not sufficient to damage neurons (at least in the doses used).

In addition, these agents are not uni-dimensional, and may have pharmacological properties that could alter the risk of neurotoxicity. For example, in some systems,

physostigmine (1-100μM) can act as a direct agonist at nicotinic acetylcholine receptors (EC_{50} 3 μM), but also as a receptor blocker at higher doses (1 mM), an effect that is in addition to its action on acetylcholinesterases (van den Beukel, et al., 1998). Such action might alter the toxicity due to muscarinic effects.

Other future experiments could include comparing the effect of these two agents in the parietal cortex, to determine if they show differences between these two regions, in parallel with MK-801. Also, although not proposed initially, the AChEI pyridostigmine bromide could be compared against physostigmine, to determine if it has a similar pharmacological profile against IPSCs in the PCC/RSC.

I. E. Differential effects of ethanol on NMDA receptor-mediated EPSCs in pyramidal cells in the posterior cingulate cortex of juvenile and adult rats (discussion of accepted manuscript included in Appendicies)

Briefly, in the attached manuscript are presented findings related to the effect of ethanol on NMDA-receptor-mediated EPSCs in the PCC of juvenile and adult rats. Previous studies had shown that ethanol possessed NMDA antagonist activity in the hippocampus (Lovinger, et al., 1989) and basloateral amygdala (Calton, et al., 1998). However, no in vitro studies had focused on the PCC/RSC regions, which may be more vulnerable to toxic effects of ethanol due to its capacity to block NMDA receptor-mediated responses.

The studies in the attached manuscript provide evidence that NMDA-receptor-mediated EPSCs in the PCC are sensitive to ethanol at concentrations within intoxicating levels in humans. Also, these studies explored the differences between juvenile and adult rats with respect to the capacity of ethanol to block NMDA receptor-mediated responses.

It was found that, at any given dose, NMDA-mediated EPSCs in slices from juvenile rats were more sensitive to the suppressive action of ethanol than responses in adults.

Discussion and future studies:

The age-related differences identified in these studies could underlie *in vivo* behavioral differences of ethanol at different ages. In addition, these findings might also predict that ethanol, when given at neurotoxic dosing regimens, might produce NMDA antagonist-related neurotoxicity that is more severe in juveniles than in adults. If so, this outcome would be the converse of the effects of other NMDA antagonists, in which younger rats are far less susceptible to the neurotoxic effects than adults (Olney, et al., 1991 & 1994).

In future studies, the methods above will be applied to the exploration of the interaction of ethanol with AChEIs, as originally proposed. In addition, although not initially proposed, these methods could also be used to explore the interaction of ethanol with the prototypical NMDA antagonist MK-801, as well as other clinically useful NMDA antagonists or AChEIs (e.g., the anti-Alzheimer's agents (Taylor, 1998). A neurotoxic interaction with MK-801 is not unlikely, for Corso et al., (1992) reported preliminary evidence that MK-801 and ethanol had a synergistic neurotoxic interaction. Specifically, neurodegeneration in the entorhinal cortex (and other olfactory-related areas) was exacerbated when MK-801 was co-administered with doses of ethanol previously shown to be non-toxic in this model (100 mg% for 4 days). In contrast, there was no exacerbation of ethanol-induced neurodegeneration after co-administration of

either DNQX (an AMPA glutamate receptor antagonist) or nimodipine (a calcium channel blocker).

Other related studies, as initially proposed, will include the 3 other non-prototypical compounds that are clinically used and that have NMDA receptor or channel blocking activity (memantine, felbamate and dextromethorphan). Additional studies not previously proposed, but relevant to this project, could include an *in vitro* study of the age-related effects of an ethanol-MK-801 interaction (or other NMDA antagonists). Given the extent to which adults (including both civilians and military populations) use ethanol (e.g., recreationally or for health-related (e.g., cardioprotective) uses, the combination of ethanol and NMDA antagonist co-exposures might be highly likely in many settings, and may be one of the most important interactions to be addressed.

I. F. Influence of animal age on NMDA antagonist-induced neurotoxicity in the PCC/RSC: possible neurotoxic mechanisms

Introduction:

Several biological parameters influence NMDA antagonist-induced neurotoxicity. There is a strong association between animal gender and animal age and NMDA antagonist-induced neurotoxicity (Olney, et al., 1989;1991; Hönack & Löscher, 1993)). Age may be an important issue with respect to this project, because most personnel involved in the PGW are adults, and the same can be expected for current and future conflicts.

However, in vitro patch clamp recordings are notoriously difficult to do in adult animals: brain slices from adult rats are much less viable that slices from young rats –

fewer cells can be seen in the living slices when placed in the recording chamber, and those cells that *can* be seen are difficult to approach with the electrode and to form the tight seals needed for patch clamp recordings. Thus, the success with which recordings can be made in slices from adults is greatly reduced compared to using slices from young animals.

Very few investigators have mastered the skills required to make these recordings, but, given the importance of the age factor to NMDA rector-mediated toxicity, we determined that the effort should be expended to attempt recording from slices from adults. Over the course of this past year, Dr. Qiang Li has developed several techniques to improve the viability of slices from adult rats, and to increase the likelihood of obtaining successful recordings. Using these techniques, he has begun an examination of the effects of NMDA antagonists on the responses recorded in male Sprague-Dawley rats of three age groups: young (postnatal day (P) 10-20), juvenile (P30-35) and adult (P130-140).

Results:

In the presence of the AMPA and GABA-A receptor blockers, unitary EPSCs were recorded from 15 pyramidal cells located in the RSC layer II and III of young, juvenile and adult rats in response to electric stimulation. Each stimulus had a duration of 0.1ms; the inter-stimulus interval was 10s. The recorded EPSCs were completely blocked by bath application of D-AP5 (50uM), indicating that EPSCs were NMDA receptor-mediated.

We first characterized the current-voltage relationship of NMDA receptor-mediated EPSCs and found that, regardless of age differences, NMDA receptors showed strong voltage dependence. Maximal responses were usually obtained when the patched cells were held at -30mV. At the membrane potential negative to –60mV, amplitudes of NMDA EPSCs were much smaller due to rectification (Data not shown).

We then test the effect of MK-801 (3uM) on postsynaptic NMDA mediated EPSCs recorded from young, juvenile and adult rats. All the experiments were performed while the membrane potential of the patched cells were held at -30mV. After 10 minutes of recording in drug-free ACSF (control), MK-801 was bath applied and the responses were continuously observed for 40 minutes. Figure-4 shows change in the peak amplitude of NMDA EPSCs, including control responses and those evoked in the presence of 3uM MK-801. Examples of recordings from slices from rats of three different ages are shown (young (A), juvenile (B) and adult (C)). In all experiments, MK-801 (3 μM) gradually reduced the amplitude of NMDA EPSCs. However, peak amplitude of postsynaptic NMDA EPSCs recorded from cortical slice of adult rat was decreased more rapidly than those of NMDA EPSCs recorded in slices from young and juvenile rats. The time required for peak amplitude of NMDA EPSCs to decrease 50% to initial value in MK-801 treatment is significantly different: 16.06 ± 1.66 for young (n=5 cells), 12.83 ± 0.79 for juvenile (n=5) and 10.17 ± 0.92 minutes (mean ± S.E.M) for adults (n=5). One-way ANOVA indicated there is a significant difference in time required for peak amplitude of evoke EPSCs to decrease to 50% of its initial value among these three groups ($F_{[2]} = 6.19143$, P=0.01421).

Summary of these changes was shown in Figure 4D. Also, responses in slices form young animals were prolonged compared to adults (Figure 5).

Discussion and Future Work:

Interestingly, the difference between the young, juvenile and adult rats was not due to an overall difference in sensitivity, but in the speed with which MK-801 exerted its effect. This could relate to the mechanism of neurotoxicity if a faster onset of the MK-801 block of NMDA receptors could prevent compensating mechanism, which might help protect vulnerable neurons (e.g., desensitization, down-regulation, or pre-synaptic inhibition of glutamate release) from being set in place. There may also be clues from our preliminary findings reported in last year's Annual Report, in which we used a photo-diode array to measure tissue-wide depolarization during MK-801 exposure. Specifically, there was a narrow time window soon after the application of MK-801 during which there was a stimulus pulse produced a much more wide-spread depolarization across the cingulate cell layers, than in control solutions, or after prolonged application of MK-801. Thus, the early stages of drug application may be important with respect to drug effect, and possibly neurotoxicity. MK-801 clearly has a very fast onset of effect, based on our in vivo studies, in which adult female rats may exhibit behavioral signs of toxicity (e.g. head weaving) within 5 minutes of an i.p. or s.c. injection.

Thus, the results obtained in this study be relevant to the age-dependent toxicity of NMDA antagonists, however, additional studies are needed to explore this issue further. Clearly, there could be many other mechanisms by which MK-801 toxicity is enhanced in adult animals compared to young animals, including differences in

metabolism and distribution of this lipophilic drug. Also, adult animals are more vulnerable to many other neurotoxins, such as, for example, the shellfish toxin domoic acid and the seaweed toxin kainic acid (Dawson, et al., 1995). These sensitivities could reflect general, cellular characteristics that render adult neurons more vulnerable to many types of neurotoxic insult, when compared to neurons from younger animals (Dawson, et al., 1995).

Future work:

NMDA receptors are composed of several subunits. Differences in sensitivity of NMDA receptor to MK-801 among the three age groups observed in our present study may reflect a developmental change in the subunits compositions of receptors. We will test this notion with ifenprodil, an NMDA receptor antagonist that preferentially blocks NR2B subunit-containing NMDA receptor.

Additional studies and analysis will be done to assess the extent to which MK-801 produces a block after the effect has reached a plateau. As shown in Figure 4 A-C, in some recordings from adults and juveniles, the block of the NMDA receptor-mediated EPSCs is near total, whereas there is some residual response in slices from young rats.

In addition, a much larger study would involve repeating the above studies using slices from young, juvenile, and adult female rats. Given that women are now serving in the Armed Forces, information about gender differences with respect to this neurotoxicity is important, especially since studies in rats have shown that adult females are the most vulnerable group to NMDA antagonist-induced neurotoxicity.

I. Electrophysiology Figure Legends

Figure 1. Photomicrographs and recording from an interneuron in the PCC/RSC showing that MK-801 blocks NMDA receptor-mediated EPSCs of PCC/RSC interneurons.

A. Photomicrograph showing interneurons (e.g., arrow) and pyramidal cells form a cortical slice (scale bar=25μm).

B. Firing pattern of an interneuron shown in A. This cell did not have frequency adaptation in response to depolarization elicited by injection of positive current to the soma.

C. With the presence of BMI 20μM, fast inward currents were dominant when the interneuron was held at –70mV. These fast inward current are sensitive to DNQX (20μM).

D. When the cell was held at +50mV, slow outward currents were recorded. MK-801 (40μM) can block these slow outward currents, indicating they are NMDA receptor mediated EPSCs.

Figure-2 Effect of pilocarpine on evoked IPSCs of PCC/RSC pyramidal cells

A. The peak amplitude of evoked IPSCs was decreased by bath application of pilocarpine (2, 5 and 10uM). The combination of pilocarpine (10μM) and MK-801 (40μM) produces further inhibition.

B. Bar graph showing the inhibitory effect on mean peak amplitude of evoked IPSCs of pilocarpine (2uμM, n=8, 5μM, n=8 and 10μM, n=8) or co-application of pilocarpine (10uM) and MK-801 (40μM) (n=3). A one-way ANOVA indicates there is a significant difference in change in peak amplitude ($P<0.05$)

Figure-3 Effect of physostigmine on evoked IPSCs of PCC/RSC pyramidal cells

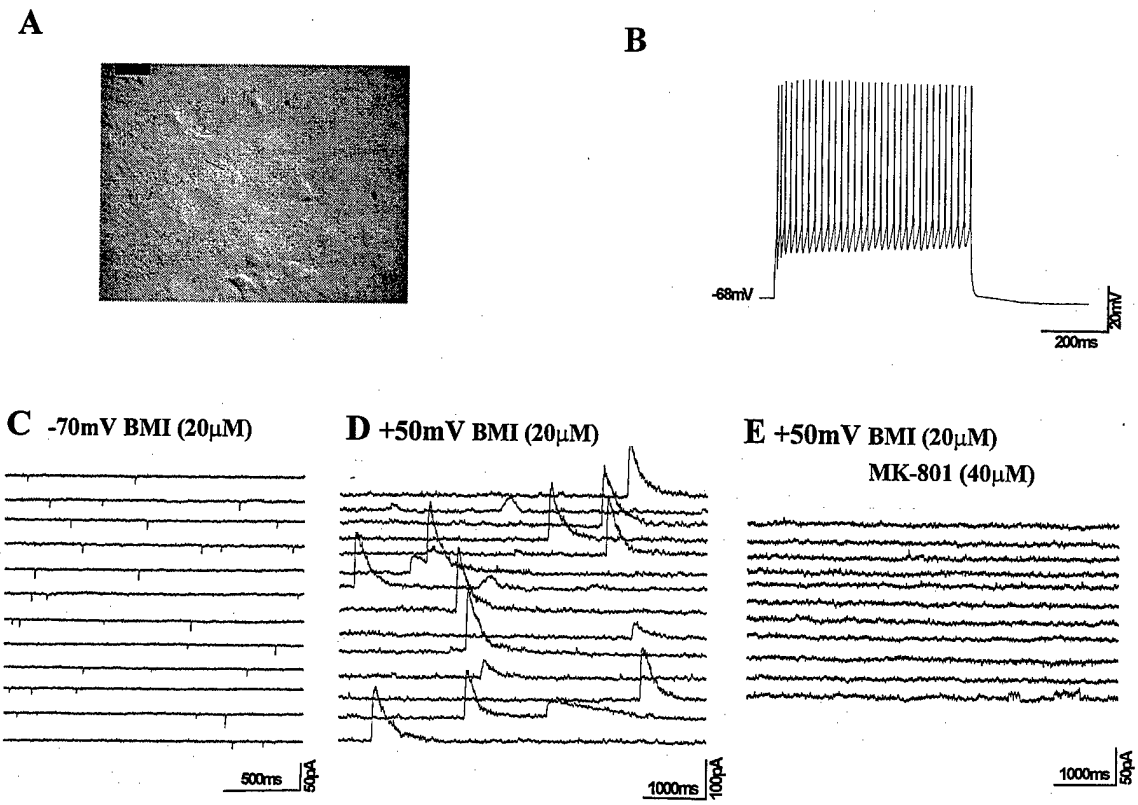
A. The peak amplitude of evoked IPSCs was decreased by bath application of pilocarpine (2, 5,10 and 100μM)
B. In another cell, co-administration of physostigmine (100μM) and MK-801 (40μM) decreased the peak amplitude of evoked IPSCs. The addition of BMI (20 μM) suppressed the remaining response, providing evidence that the response was mediate by GABA-A receptor activation.
C. Bar graph shows inhibition of physostigmine {(2μM (n=6); 5μM (n=6); 10μM (n=6) and 100μM (n=3)} and co-application of physostigmine (100μM) and MK-801 (40μM) (n=3) on mean peak amplitude of evoked IPSCs. One-way ANOVA indicates there is a significant difference in change in peak amplitude ($P<0.05$).

Figure 4. Age-dependent sensitivity of NMDA receptor-mediated EPSCs to MK-801. NMDA receptor-mediated EPSCs were isolated using the GABA-A receptor blocker BMI (20uM) and the AMPA receptor blocker DNQX (20uM). The cell membrane potentials were held at –30mV throughout the experiments.

(A, B & C) Graphs show three examples of NMDA EPSCs recorded from young (A), juvenile (B) and adult (C) rats. Data points represent peak NMDA receptor-mediated EPSC amplitudes before and after bath application of MK-801 (3uM).

(D) Bar graph shows mean time required for peak amplitude to decrease to 50% of its initial value in young (n=5), juvenile (n=5) and adult (n=5 cells).

Figure 5. Age-related effects on the duration of MDA receptor-mediated EPSCs.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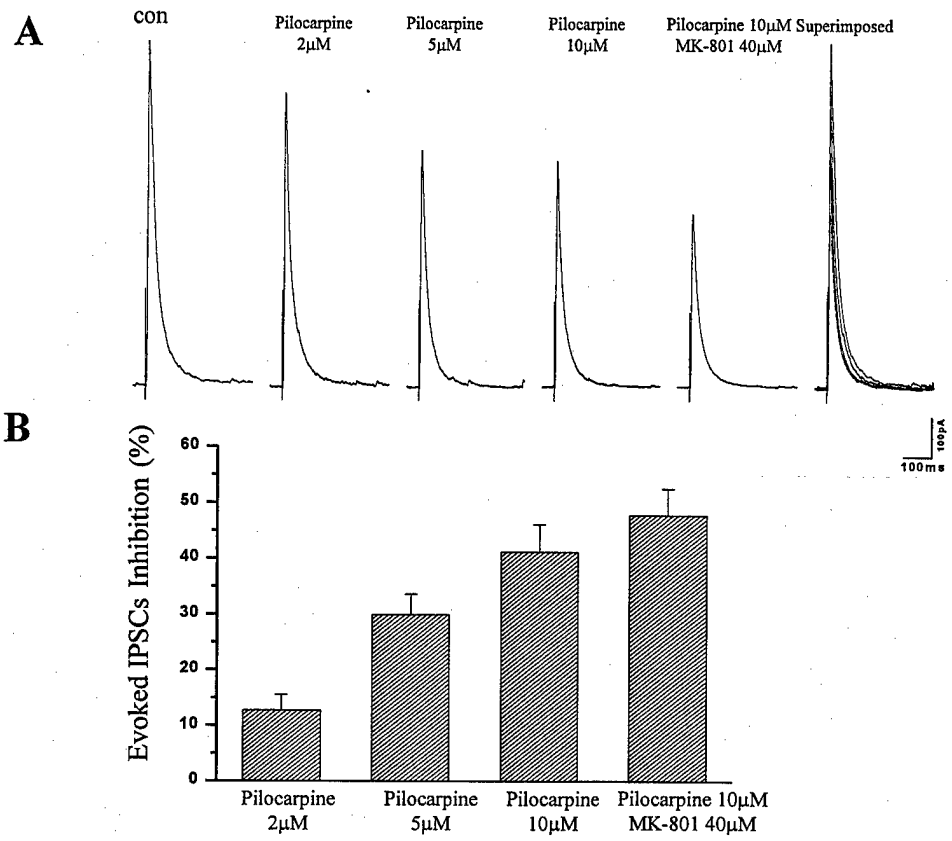
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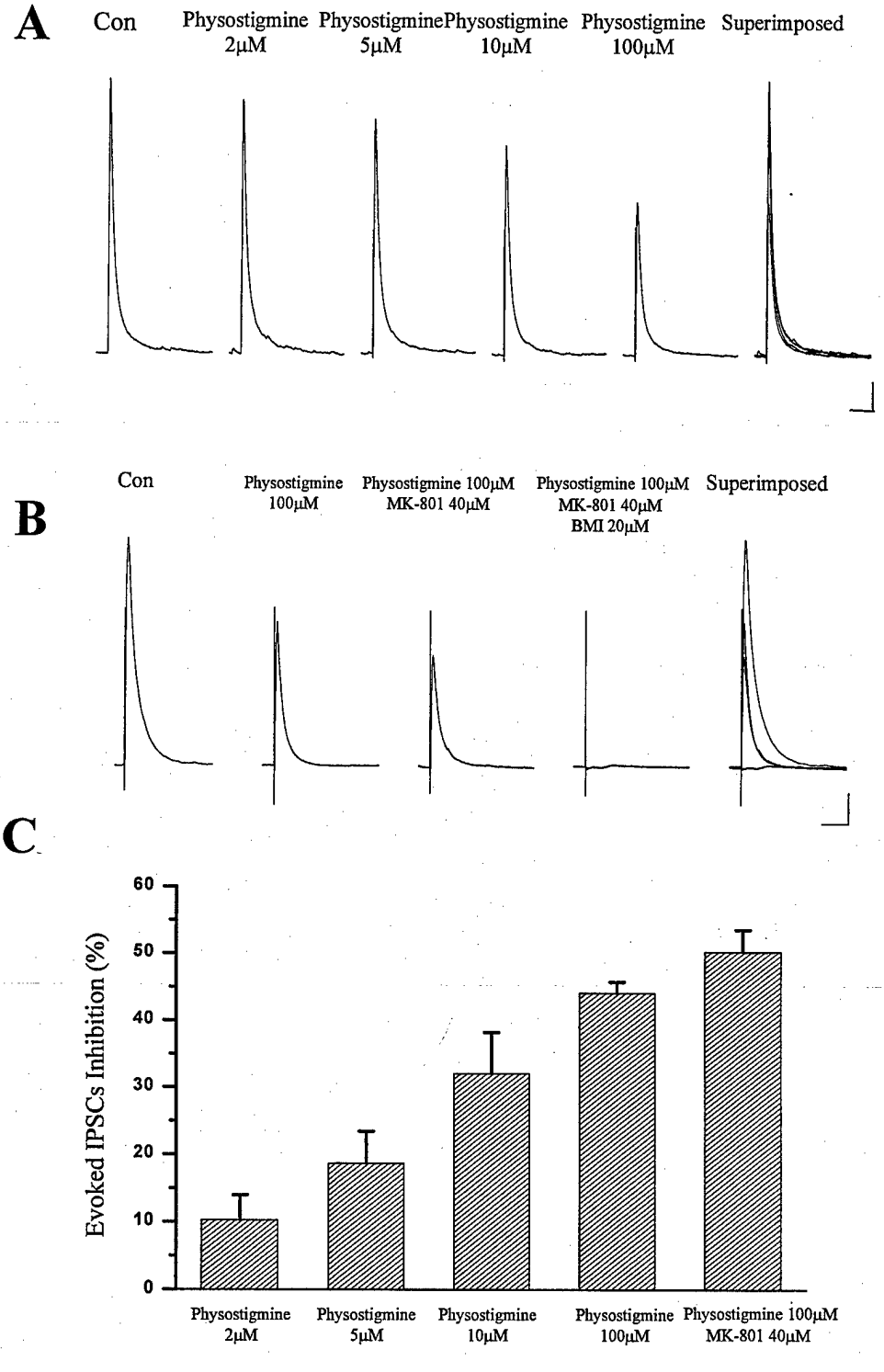
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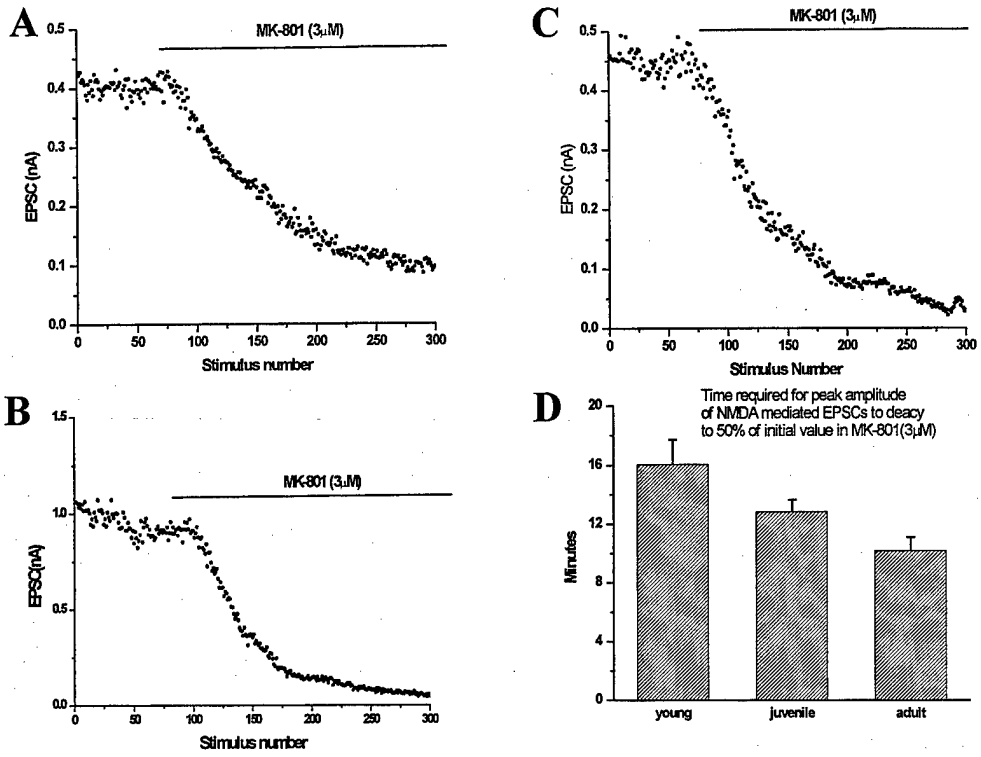
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Stimulus number
C
MK-801 (3μM)
EPSC (nA)
Stimulus Number
D
Time required for peak amplitude
of NMDA mediated EPSCs to deacy
to 50% of initial value in MK-801(3μM)
Minutes
young
juvenile
adult

NMDA EPSC Duration

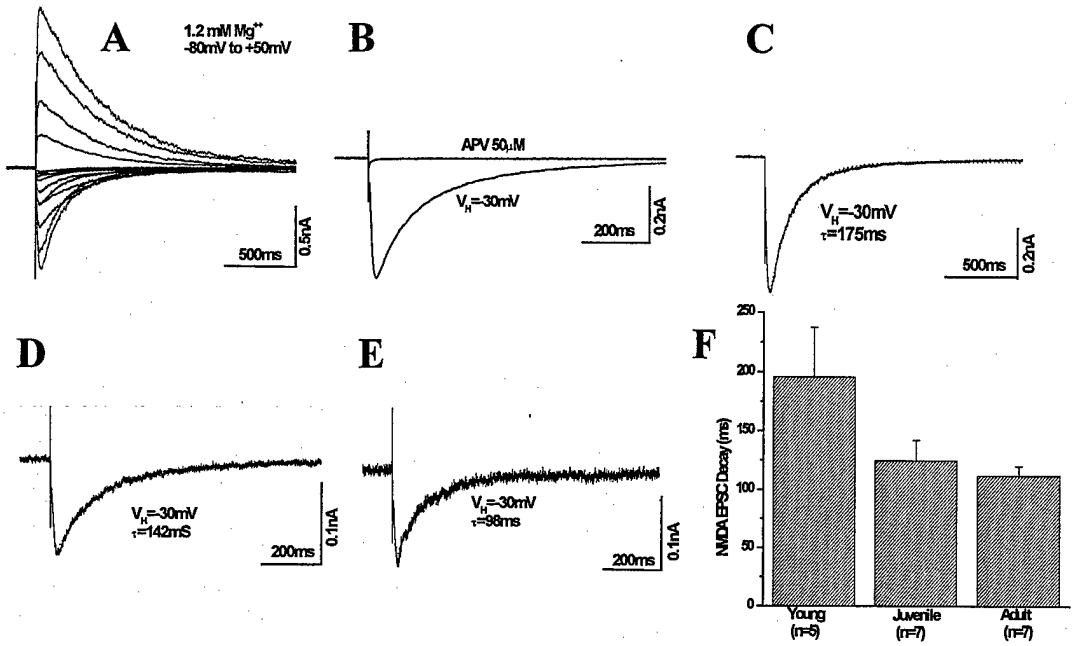


Figure-5. Developmental decrease in NMDA-EPSC duration. In the presence of BMI (40uM) and DNQX (20uM), NMDA receptor mediated EPSCs were isolated. A. Voltage dependent of EPSC. B. EPSC was blocked by APV(50μM). EPSCs recorded at holding potential of –30mV from young (C), juvenile (D) and adult (E) rats fit with a single exponenti Each trace is an average of 3 responses. F. Bar graph shows decrease in the mean decay time of NMDA-EPSCs. Signific difference at $P<0.05$ (One-way ANOVA).

PART II. In Vivo Studies: behavioral monitoring and histopathology.

PART II-A. In vivo exposures to NMDA Antagonists and Acetylcholinesterase Inhibitors in the Rat Cingulate Gyrus and Retrosplenial Cortex: Overview

PART II-B. Neurodegeneration detected using Fluoro-Jade B (FJ-B) staining in the PCC/RSC due to exposure to NMDAr/c antagonist and AChEIs.

PART II-C. Functional Observational Battery (FOB) Behavioral Test in rats exposed in vivo to NMDAr/c antagonists and AChEIs.

PART II-A. In vivo exposures to NMDA Antagonists and Acetylcholinesterase Inhibitors in the Rat Cingulate Gyrus and Retrosplenial Cortex: Overview

In the first year of study we established successful histopathology methods using Fluoro-Jade staining (versus counting vacuoles using H & E stains) and determined optimal doses of the drugs to be most relevant to clinical exposures. Our preliminary results suggested that there may have been an interaction between MK-801 and pyridostigmine bromide, but we did not have sufficient numbers for statistical analysis.

In the second year of this project, we have adapted an expanded method for assessing behavioral changes (a Functional Observational Battery (FOB)), and also began to use the new fluorochrome Fluoro-Jade B to assess the neurotoxicity of the NMDA antagonists MK-801 alone or in combination with two AChEIs, pyridostigmine (PB) and physostigmine.

Behavioral testing has been greatly expanded over the original proposal. This was done to take advantage of the prolonged behavioral changes that occur for several days after injection. Specifically, after we switched from counting vacuole-containing neurons using H & E staining (for which rats were sacrificed after only a 4 hour delay) to Fluoro-Jade staining, the additional days of recovery (3 days produce optimal Fluoro-Jade staining) provided a window in which to assess the longer lasting effects of the drugs. This change will hopefully provide more information about lasting effects of the NMDA antagonists and AChEIs, and the ability of other agent to exacerbate or improve the response, and also provide a baseline if more chronic studies are of interest in the future.

We changed from the first generation Fluoro-Jade stain to the new stain, Fluoro-Jade B (FJ-B), because this later stain has proved to be superior to the standard Fluoro-Jade stain we used in the first year of study. Fluoro-Jade was the first generation stain in this class, and whereas it was stable, reliable and reproduceable (with good agreement between individuals counting degenerating cells) there was enough background staining in many sections so that photographic images were not optimal. With the availability of this new generation stain (Fluoro-Jade-B), the cells stand out against a darker background, facilitating the counting of positive neurons, as well as photography (Schmued & Hopkins, 2000). .

Breifly, as discussed above, these studies were done to assess the interaction between NMDA antagonists and AChEIs. Olney first reported that non-competitive NMDA antagonists produce vacuoles in neurons of the posterior cingulate (PC) and retrosplenial cortex (RSC) within four hours of exposure, but the effect appeared to be reversible (Olney, et. al., 1989). This reversibility suggested that neurons would recover eventually. Then, in 1991 they reported that higher doses of NMDA antagonists could produce permanent neurodegeneration, as seen using sliver neurodegeneration stains (Olney, et al., 1991). The seriousness of this effect is new well-recognized, and has been reproduced elsewhere (Auer, 1994; Ellison, 1995; Hargeaves, et. al, 1993; Schmued, et al, 1997; Schmued & Hopkins, 2000).

NMDA antagonist-induced neuronal degeneration can be attenuated by modulators of many other neurotransmitter systems. It can be exacerbated by the muscarinic cholinergic agonist pilocarpine (Corso, et al., 1997), or blocked by atropine (a muscarinic cholinergic antagonist), suggesting that cholinergic processes modulate

NMDA antagonist-mediated neurotoxicity (Olney, 1991). Barbiturates and benzodiazepines were also protective (Olney, 1991), as were alpha-2 adrenergic agonists (Farber, et al., 1995) and antipsychotics (Farber, et al., 1996; Sharp, et al., 1993; 1994) and SSRI antidepressives (Tomitaka, et al., 2000).

However, with respect clinical significance, the exacerbation by pilocarpine is a troubling finding, especially if AChEI's had a similar effect. Given the risk that both military personnel and civilians could be exposed to both NMDA antagonists and AChEIs, we began the following studies.

Discussed below are methods and preliminary findings from histological and behavioral studies that evaluate potential neurotoxic interactions between these agents.

Methods:

Rats:

Rats were housed in the Durham Veterans Affairs Vivarium under the care of Dr. Gerald Olsen, kept on a 12 hour light and dark cycle, and allowed access to food and water. All efforts were made to reduce animal stress and suffering, in accordance with the *Guide for the Care and Use of Laboratory Animals* (1996) and the current *Durham VA Medical Center Manual for Animal Research,* and all efforts were made to reduce the number of animals needed, while maintaining statistical validity.

Our methods were similar to those used by Olney and colleagues with respect to rats (age and gender), drug doses (MK-801), and behavioral observations (Olney, et al., 1991; Corso, et al., 1997). We used Sprague Dawley (S-D) rats from Charles River (Raleigh, NC). Rats used were adult female retired breeders, which represent the most sensitive age and sex with respect to NMDA-antagonist-induced toxicity.) The adult

female rats were ordered directly from Charles River or were retired breeders delivered to our facility with litters of 14-day-old pups. Litters were weaned at ~ 28 days of age, after which time the dams recovered at least 4 weeks. The exact age of retired breeders is not provided by Charles River, but the rats are full-sized adults when used (300-500 g). This assures that we are have animals well within the range previously reported as being most sensitive to neurotoxicity.

Injections and Drugs:

Rats were randomly assigned to a control or treatment group. Rat were weighed before experiments, then given drugs (or vehicle), intra-peritoneal (ip) or subcutaneous (sc), in a volume of 1ml/kg. In rats given 2 different drugs, MK-801 was given first, after which the AChEI was given within 15 minutes. Drugs were prepared fresh each day and dissolved in sterile saline for injection. The (-) physostigmine sulfate was protected from light; (-) physostigmine sulfate was checked for light-induced degradation (red color).

The following drug doses were given: (+)MK-801 (0.3-1 mg/kg); (-) physostigmine sulfate (0.03-1.0 mg/kg), and pyridostigmine bromide (0.1 & 0.3 mg/kg). (The ED_{50} for producing vacuoles detectable by light microscopy PS/RSG cortices in adult female S-D rats was (0.18 mg/kg sc) (Olney, et al., 1989); higher doses (5.0 mg/kg sc) produce severe vacuole reaction in the cingulate and R/S cortex (Olney, et al., 1991).) Doses were chosen to avoid lethal interactions. For example, the pyridostigmine dose was 0.1 mg/kg. This moderately low dose has been used in rats (Domino, 1987) and also in guinea pigs (Berry and Davies, 1970), the latter in which it was found to be a "maximum sign-free dose". We specifically wanted to use modest doses of

pyridostigmine and MK-801, because we want to "model" a clinically reasonable scenario.

After injections, each rat was placed in a clear plastic cage and observed for drug-induced changes in behavior (see FOB methods below). Because agents which increase cholinergic drive can produce severe seizures at high doses (Turski, et al., 1989), rats were monitored for behavioral evidence of status epilepticus (continuous rapidly recurring seizures), because, on their own, such seizures can cause neuronal death or damage. This has not happened yet, but any rat found in status epilepticus will be euthanized. Dosing was adjusted if necessary (e.g., when too high given in combination).

Perfusion and Histopathology:

After 3 days, rats were anesthetized with Halothane and transcardially perfused with heparinized saline followed by 4% paraformaldehyde. Brains were removed immediately and post-fixed overnight or, in some cases the perfused rat was refrigerated overnight and the brain removed the next morning. (The latter was suggested by R. S. Sloviter for vibratome, next-day sectioning). Brains were transferred to 0.1 M phosphate buffer (PB) for one day, then to 30% sucrose for 3 to 4 days, until they sink. Cryostat sections were cut (40 μm), then stored in 0.1 M PB at 4° C. Sections were processed for Fluoro-Jade staining as described (Schmued, et al. 1997) or Fluoro-Jade-B staining (Schmued, & Hopkins, 2000a & 2000b). Sections were mounting in distilled water on gelatinized slides, dried over night, and processed in the following solutions: 100% EtOH (3 min); 70% EtOH (1 min); distilled water (1 min); 0.06% potassium permanganate (7.5 min with slow shaking); distilled water (1 min); 0.001% (Fluoro-Jade) or 0.0004% (Fluoro-Jade-B) working solution (30 min with slow shaking in the dark); distilled water

(3 times for 1 min), then dried in dark for at least 2 hours (but ≤2 days), dehydrated in alcohol and xylene, then coverslipped with D.P.X., Sections were examined with an epifluorescence microscope using a FITC filter. Assessment of neurotoxicity was performed by an individual blinded to treatment. Bilateral cell counts were made from 4 sections from the posterior cingulate and 4 from the retrosplenial cortices. From Sigma/RBI (St. Louis, MO) we obtained (+)MK-801, (+)pilocarpine HCl, pyridostigmine bromide, (-) physostigmine sulfate; chemicals were from Sigma or Mallinckrodt. Fluoro-Jade and Fluoro-Jade B were obtained from Histo-Chem, Inc. (Jefferson, AR).

PART II-B. Neurodegeneration detected using Fluoro-Jade B (FJ-B) staining in the PCC/RSC due to exposure to NMDAr/c antagonist and AChEIs

Results:

As reported previously, MK-801 produced neurodegeneration in the PCC/RSC, whereas pyridostigmine alone did not. We now report that physostigmine also did not produce neurodegeneration when used alone. Figure 1 shows a photomicrograph of the areas of interest (PCC/RSC). Figures 2-5 provide examples of Fluoro-Jade-B staining in sections from rats given saline or MK-801 plus-or-minus pyridostigmine or physostigmine. At this point, we have given doses of saline or 0.3 mg/kg MK-801, 0.1, 0.3, and 1.0 mg/kg PB, and 0.1 or 0.3 mg/kg physostigmine to a total of 70 rats, and are in the process of blinded cell counting and data analysis, on which results and conclusions are pending. Given the occasional variability that we have seen with MK-801 alone, the final studies may require larger n's than at first was anticipated. Thus,

additional groups of animals are being exposed to verify the interaction of MK-801 and AChEIs.

PART II- C. Functional Observational Battery (FOB) Behavioral Test in rats exposed in vivo to NMDA antagonists and AChEIs

FOB Methods

Overview:

Behavioral tests were administered to each animal prior to drug exposure (pre-drug), and additionally at one, four, twenty-four and forty-eight hours after the injection (post-drug). The rating observer was blinded to treatment. The observer began the test panel (Table 1) by observing each rat in its home-cage environment (clear plexiglass cage), and then by observing the animal after it was removed to another cage for observation and manipulation (the arena). The FOB assessments were based on previously reported tests (Annon., 1998; Gad, 1982; Irwin, 1968, Moser, 1996; Moser, et al., 1988; Tilson & Moser, 1992), with adjustments made for specific behaviors associated with MK-801 exposure (e.g., head weaving).

Home-cage observations:

Each home-cage observation was performed in the same sequence, beginning with an unobtrusive assessment of the animal's body posture, bizarre behaviors, tremors, twitches, tonic or clonic convulsions, exophthalmos, and eye-crustiness. Body posture was rated on a scale of 1-10, with normal alert behavior scored at 1, and 10 describing a rat that is lying completely flat. Any bizarre behavior observed was given a score between 1-3 based on the degree of severity with a three as most severe. A brief

description of the bizarre behavior was also noted. (Both head-weaving and aimless wandering were typical bizarre behaviors that were observed after MK-801 treatment.) Tremors and twitches were also both scored (1-5); tremors were further described as exertion, head or tail tremors. Severe tremors or twitching rated higher scores. Convulsions were rated as either clonic or tonic and scored descriptively within those categories (between 1-3 for clonic convulsions and 1-5 for tonic convulsions). For example, clonic chewing behavior (scored as a one) is described as less severe than repetitive whole-body clonic tremors (scored as a three), and tonic opisthotonus (scored as a two) was described as less severe than popcorn convulsions (scored as a four). Finally, the presence of either exophthalmos or eye-crustiness was scored with a one.

Novel cage observations:

After completion of the home-cage observations, the observer would remove each rat, weigh it (once each day of the study), then transfer the rat to a fresh cage to record the following: the animal's state of arousal when removed from the home-cage (scored between 1-6, with a one describing a limp, easy to handle rat, and a five as a rat that is aggressive or otherwise difficult to remove from it's home-cage). The presence of convulsions or tremors as a result of being handled, and the degree of palpebral closure were all scored as described above. The latency (seconds) before the animal's first step, whether or nor the animal exhibited piloerection, and general fur appearance were all recorded, as well as the total number of rears, grooming episodes, and the degree of tail elevation. The observer then began to manipulate the rat to assess: the degree of startle response elicited by a single clap (1-3, a score of two is a normal response, a score of one is no response), provoked biting elicited by placing a pencil in the rat's mouth (scored 1-

5), the rat's reaction to the presence then approach of the observer's finger near the animal's head (also scored 1-5), and the rat's reaction to touch on the hindquarters (scored 1-7, with a one describing a none-responsive rat, and a seven a rat that leaps away from the observer's touch. Moderate scores from these evoked behaviors describe normal responses. Finally, the rat's gait, total gait incapacity and the degree of the animal's limb rotation (from normal stance) are recorded. Gait scores range from 1-6, with a one describing normal gait; two, a slightly ataxic gait; and six, a rat that cannot walk. Total gait incapacity describes the ability of the rat to move around despite any gait disability, gait incapacity scores range from 1-4 with a one describing normal locomotion, and a four a rat that is totally impaired and cannot walk. Limb rotation scores range from 1-5 with one describing a normal rat with no limb rotation to a five, which describes a rat with severe limb rotation.

The rats were handled by the tail to assess their degree of positional passivity (or struggle during tail suspension), visual placing, grip strength, and pencil grip. Each of these behaviors was scored by degree of severity between 1-5, (or 1-3 for the pencil-grip test). Moderate scores described normal rat behaviors. Visual placing was assessed by holding the rat suspended above the arena cage floor and then lowering the rat to the cage floor while observing the rat's forearm extension. A normal rat was scored with a value of four and displayed forearm extension well before the animal's vibrissae contacted the cage floor. Grip strength was scored as the animal's ability to grasp and hold onto the edge of the arena cage. The pencil grip tested the animal's ability to grasp a pencil placed in front of the rat as it was suspended by the tail above the cage floor. The presence of hypothermia or lacrimation was recorded with a value of one. Each animal

was tested for extensor thrust by holding the rat by the tail close to the arena cage bottom, and placing a hand on the pads of the animal's rear feet. Scoring ranged between 1-4. An unimpaired rat exhibits a strong push of the hind limbs against the observer's hand, and would thus be given a score of one. The non-responding, impaired rat would receive a score of four.

Pain responses were determined by quickly pinching the rats hind limb toes, and their tails with blunt forceps and recording the response. The toe-pinch was scored between 1-5, and the tail-pinch between 1-7. Higher scores describe animals that are quite sensitive to either stimulation.

Righting reflexes were determined by flipping the animal onto it's back and recording the degree of impairment exhibited by the animal in righting itself. Similarly, the catalepsy score was determined by placing the animal's hindquarters onto a flat box 1.5 inches high in the arena cage, and recording the animal's ability to remove itself from that position. Both righting reflex and catalepsy scores range between 1-4, with a one describing the unimpaired rat, and a four a very impaired rat.

Finally, the presence of diarrhea and the degree of salivation were recorded; the animal's degree of irritability in being handled, and it's tendency to freeze were also scored. The total number of vocalizations and the number of fecal boluses deposited in the arena cage within the three-minute test period were recorded at the conclusion of the functional observation battery.

The quantitative and descriptive data acquired during the functional observation battery were analyzed by grouping the behavioral observations into seperate functional domains (Table 2).

Results:

Figures 6-18 provide examples of the behavioral responses of rats to saline or MK-801 (0.3 mg/kg), plus-or-minus PB at doses of 0.1-1.0 mg/kg. Although any final conclusions based on the results are pending additional experiments and analysis, there appears to be an effect of MK-801 and PB on several measures that are detected acutely, and some that appear to persist beyond the first day. Although some measures suggest the two agents may interact, it remains to be determined whether these changes are statistically significant.

Future Studies:

Now that both the behavioral assessment (using the FOB) and the histopathology methods (Fluoro-Jade-B), and drug doses have been optimized, we will proceed with the studies outlined in the proposal. We will finish testing the two protypical AChEIs, and then test the range of other NMDA antagonists in this model. These proposed agents include drugs most likely to be encountered clinically: memantine (25, 50, and 75 mg/kg), felbamate (100, 300, 400 mg/kg), and dextromethorphan (10, 20 and 50 mg/kg). Later studies will involve experimental agents, such as the non-competitive antagonists, as well as antagonists of the strychnine-insensitive glycine site on the NMDA, some of which may pose less of a neurotoxic risk (Koek & Colpaert, 1990; Auer, 1997; Hawkinson, et al, 1997; Tomitaka, et.al, 1996) than the NMDA channel blockers (e.g., see Berger, et al., 1994). The majority of these behavioral and histopathological studies

remain to be done, but the techniques developed over the past year will provide more information and clearer outcomes than those originally proposed.

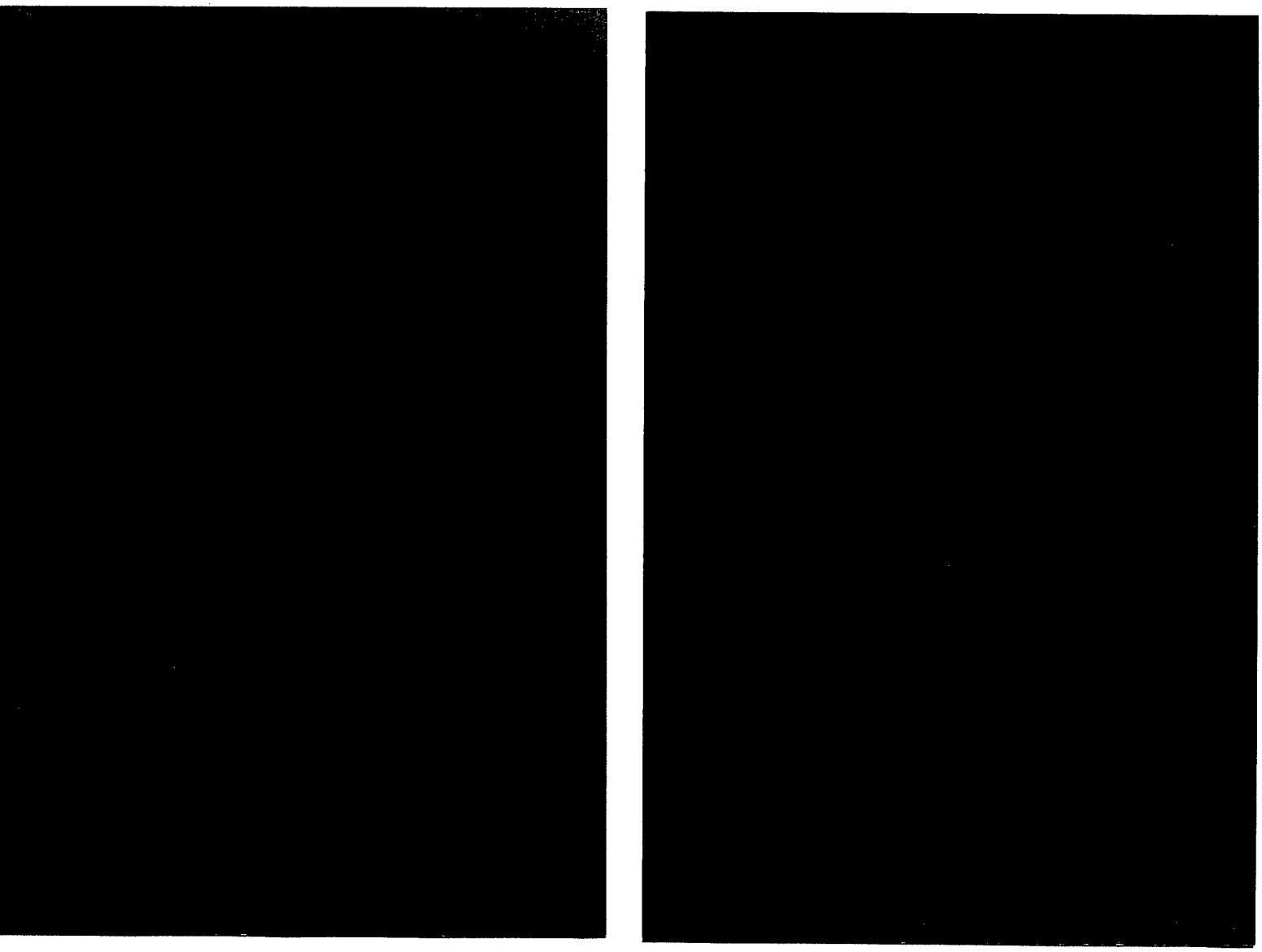
Part II Figures Legends: Histopathology and Behavioral Assessment

Figures 1-5: (The legends are included on these Figures)

Figures 6-18: Graphs of FOB behaviors measured - grouped according to system effected - in rats exposed to saline or MK-801 plus-or-minus pyridostigmine bromide. (See above methods for details of texts included in the different subgroups.)

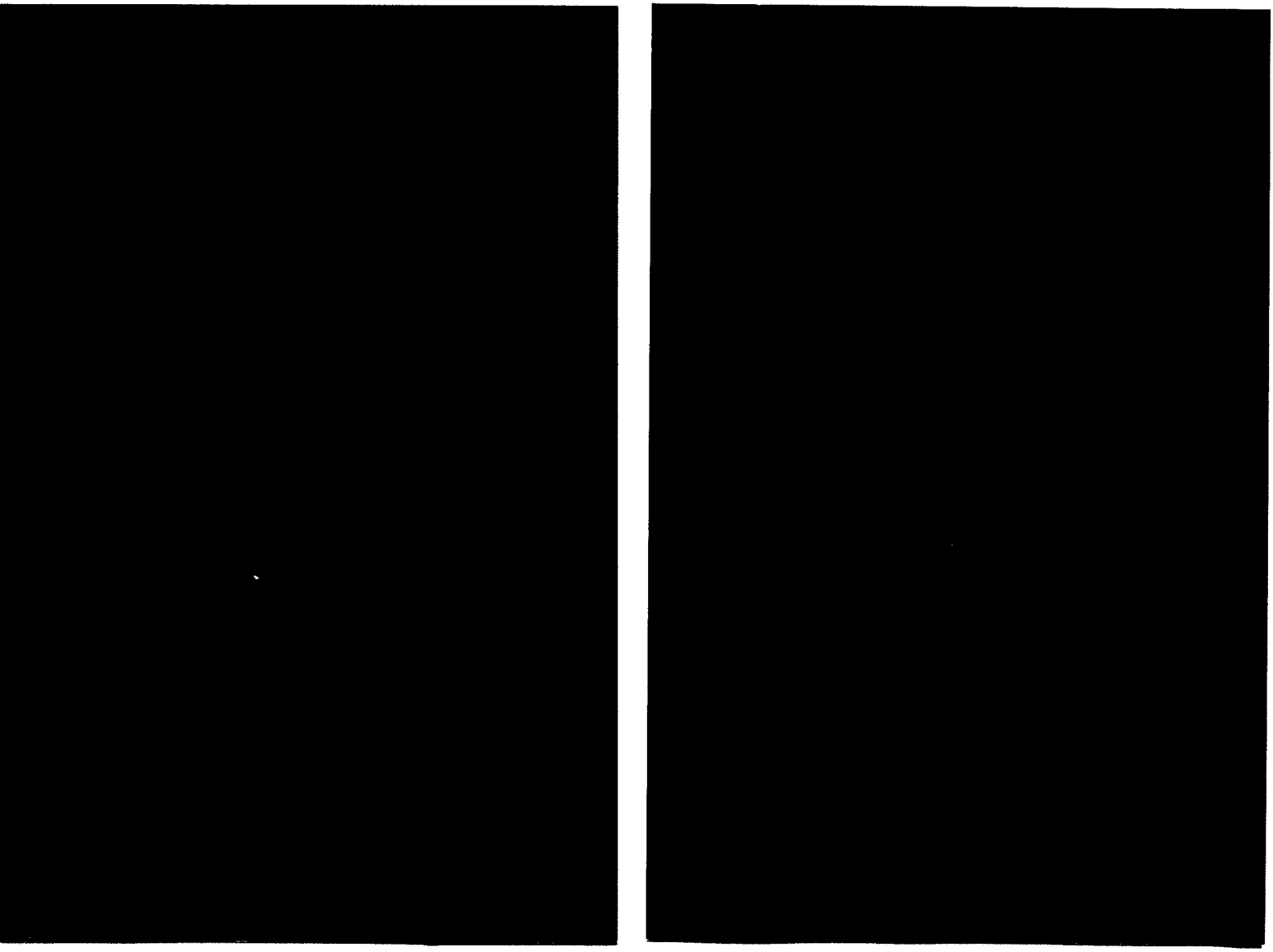
Table 1: Animal weights before and after drug exposure.

Table 2: Sample score sheet for FOB.



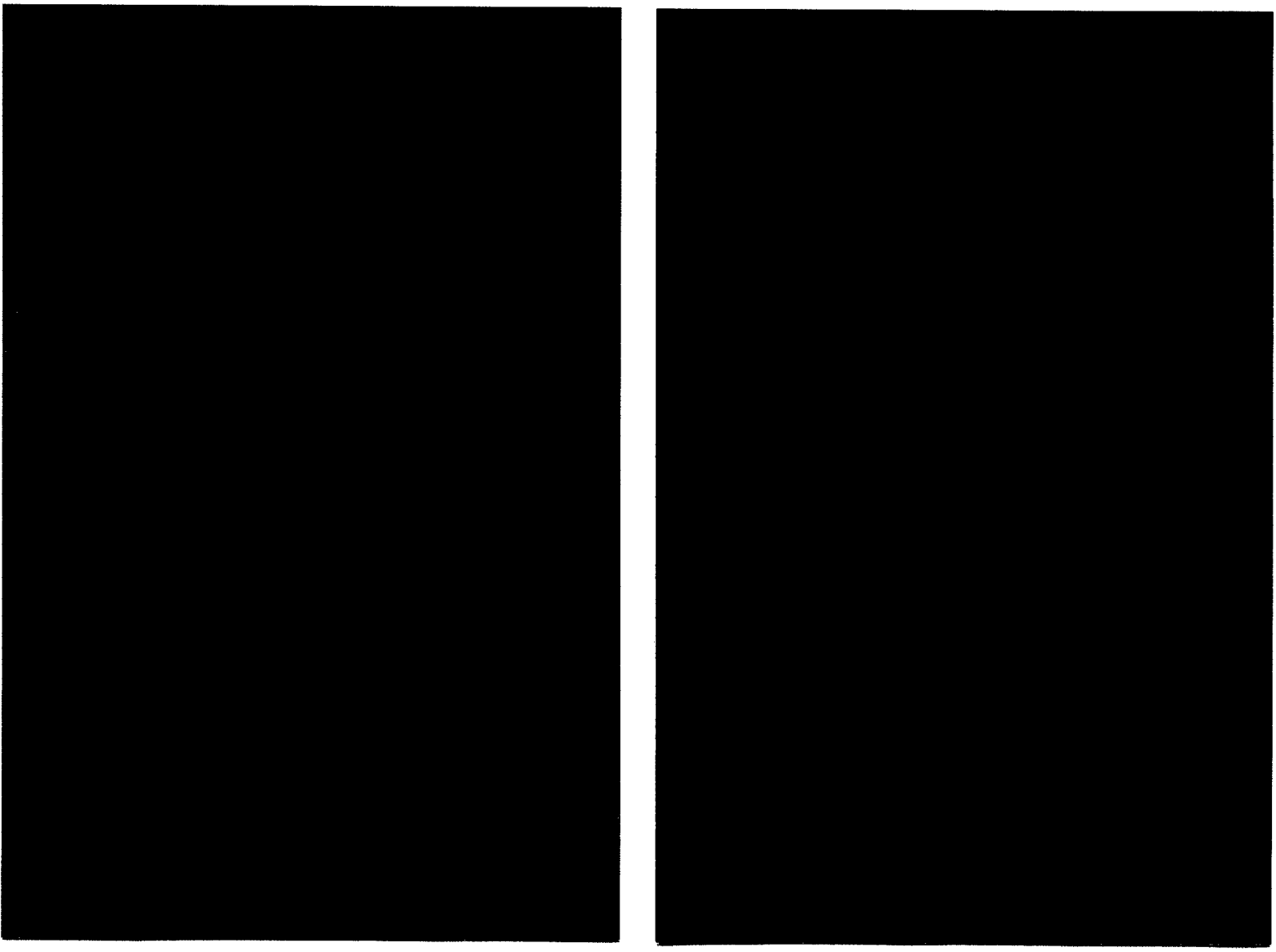
A B

Figure 1: Anatomical Areas of Vulnerability
Brain section of a Fluoro Jade B (FJ-B)-stained section shown at two magnifications. Both images were acquired using visible light. The images on the left and right were taken with a 10X and 20X objective, respectively. Arrows indicate the region in which bilateral FJ-B-positive cells were counted (i.e., depending on the rostral-to-caudal orientation, the posterior cingulate cortex and retrosplenial cortex (PCC and RSC) (Paxinos and Watson, 1986).



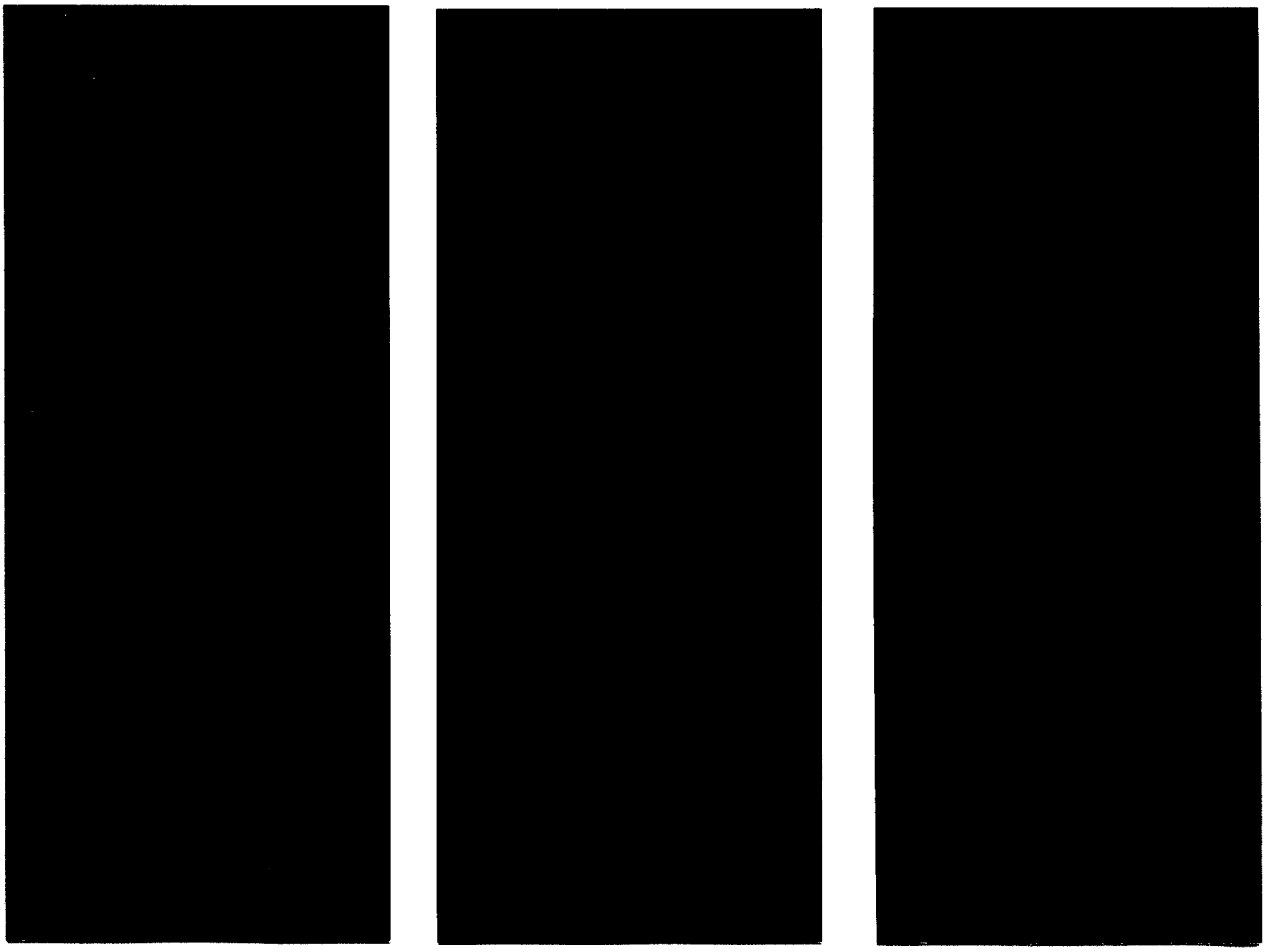
A B

Figure 2: Control (saline) and MK-801
Fluoro Jade-B-stained sections from rats injected with control solutions (saline + saline) (left), or MK-801 (MK-801 (0.3 mg/kg) + saline) (right). No FJ-B-positive cells are evident in the section from the saline-treated control rat, whereas many FJ-B-positive cells are seen in the section from the MK-801-treated rat, evidence that MK-801 produces neurodegeneration, even at this low dose. Note that in some cells, FJ-B-positive processes can be seen projecting from the cell body. In both images, the PCC/RSC is shown with the pial surface oriented to the top. All images in this figure (and subsequent figures) were taking using the 20X objective. Both rats (and all rats in subsequent examples) were injected 3 days before perfusion fixation (a point of high FJ-B-positive staining).



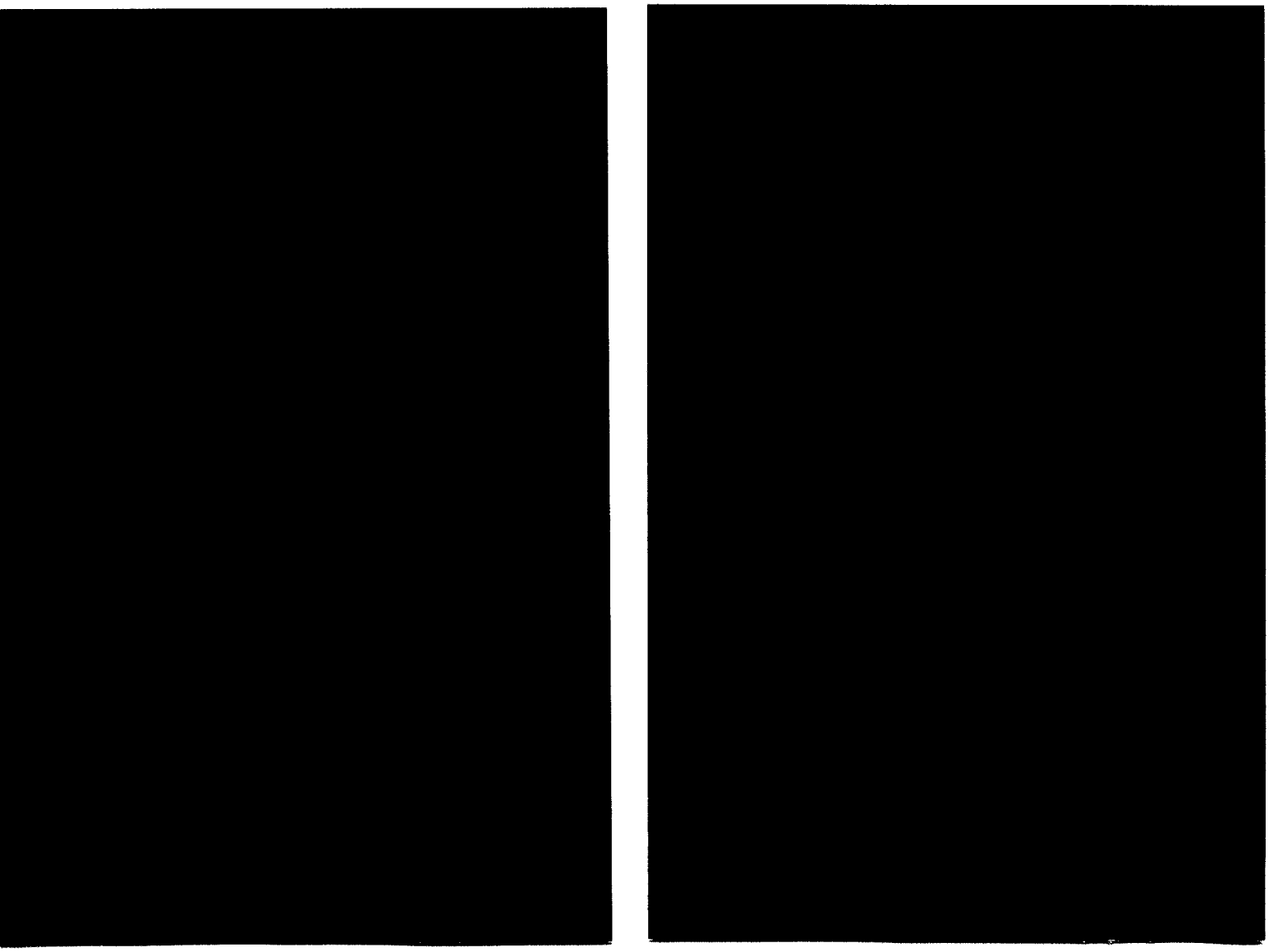
A B

Figure 3: Pyridostigmine bromide (PB) and Physostigmine
Fluoro Jade-B-stained sections from rats injected with saline + pyridostigmine bromide (1.0 mg/kg) (left) or saline + physostigmine (0.3 mg/kg) (right). No FJ-B-positive cells are evident in either section, suggesting that at these doses, neither drug produced neurodegeneration in the PCC/RSC. Images are oriented and magnified as shown in Figure 2.



A B C

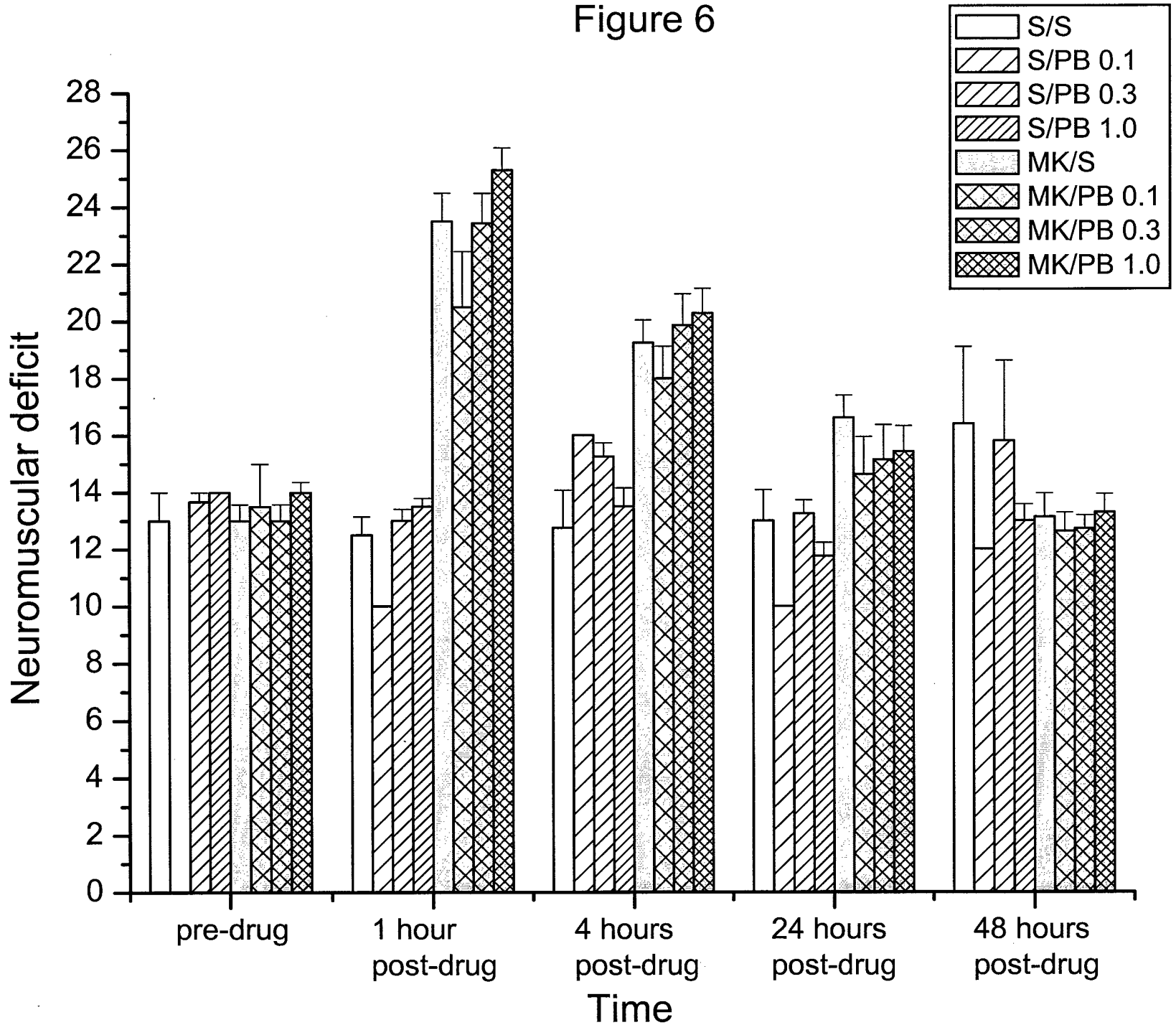
Figure 4: MK-801 and Pyridostigmine Bromide (3 doses)
Fluoro Jade-B-stained sections from rats injected with MK-801 (0.3 mg/kg) + pyridostigmine bromide at 3 different doses (in mg/kg): 0.1 (left), 0.3 (center), and 1.0 (right). Images are oriented and magnified as shown in Figure 2.



A B

Figure 5: MK-801 and Physostigmine (2 doses)
Fluoro Jade-B-stained sections from rats injected with MK-801 (0.3 mg/kg) + physostigmine at 2 different doses (in mg/kg): 0.1 (left) and 0.3 (right). Images are oriented and magnified as shown in Figure 2.

Figure 6


S/S
S/PB 0.1
S/PB 0.3
S/PB 1.0
MK/S
MK/PB 0.1
MK/PB 0.3
MK/PB 1.0
Neuromuscular deficit
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
pre-drug
1 hour post-drug
4 hours post-drug
24 hours post-drug
48 hours post-drug
Time

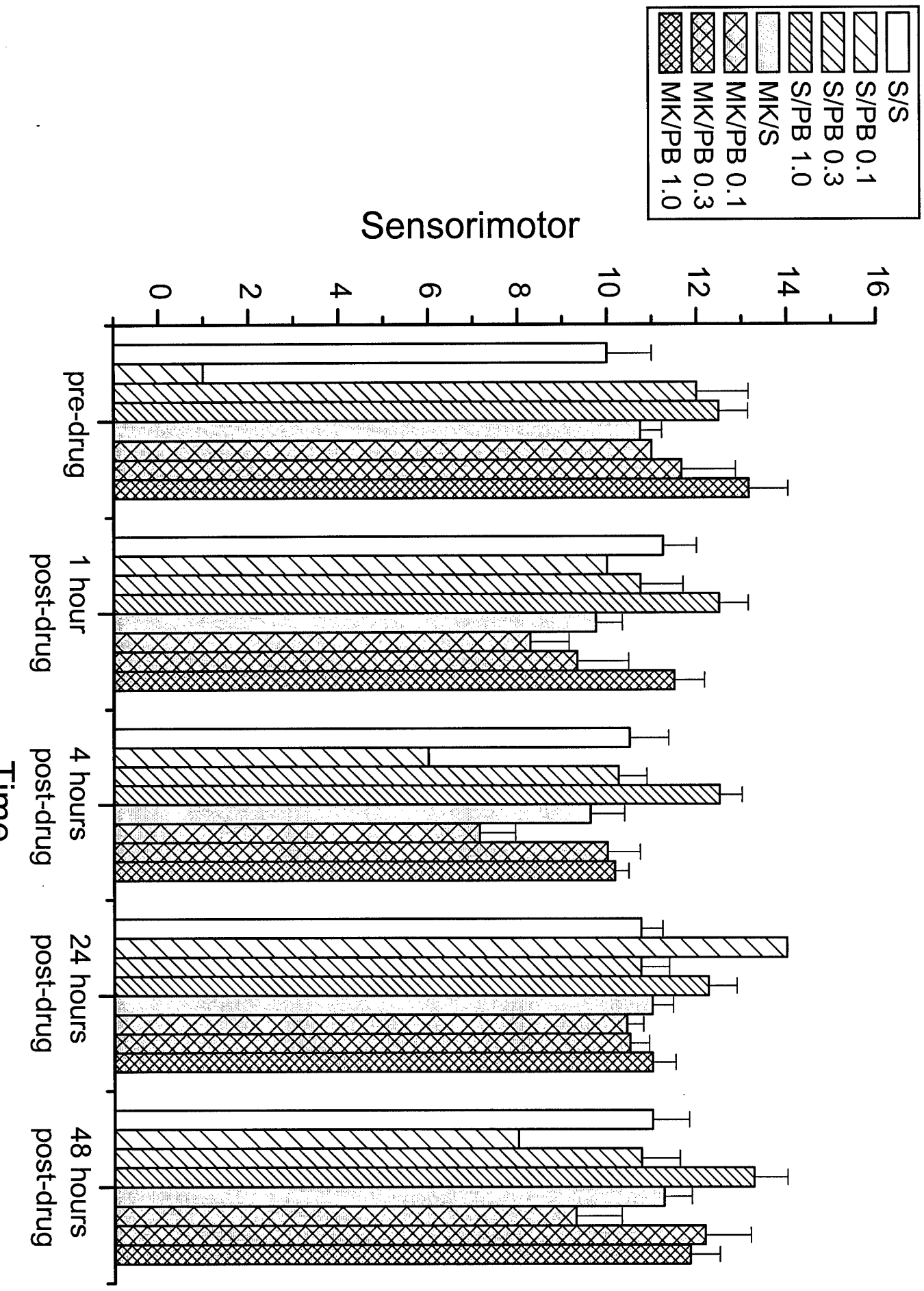
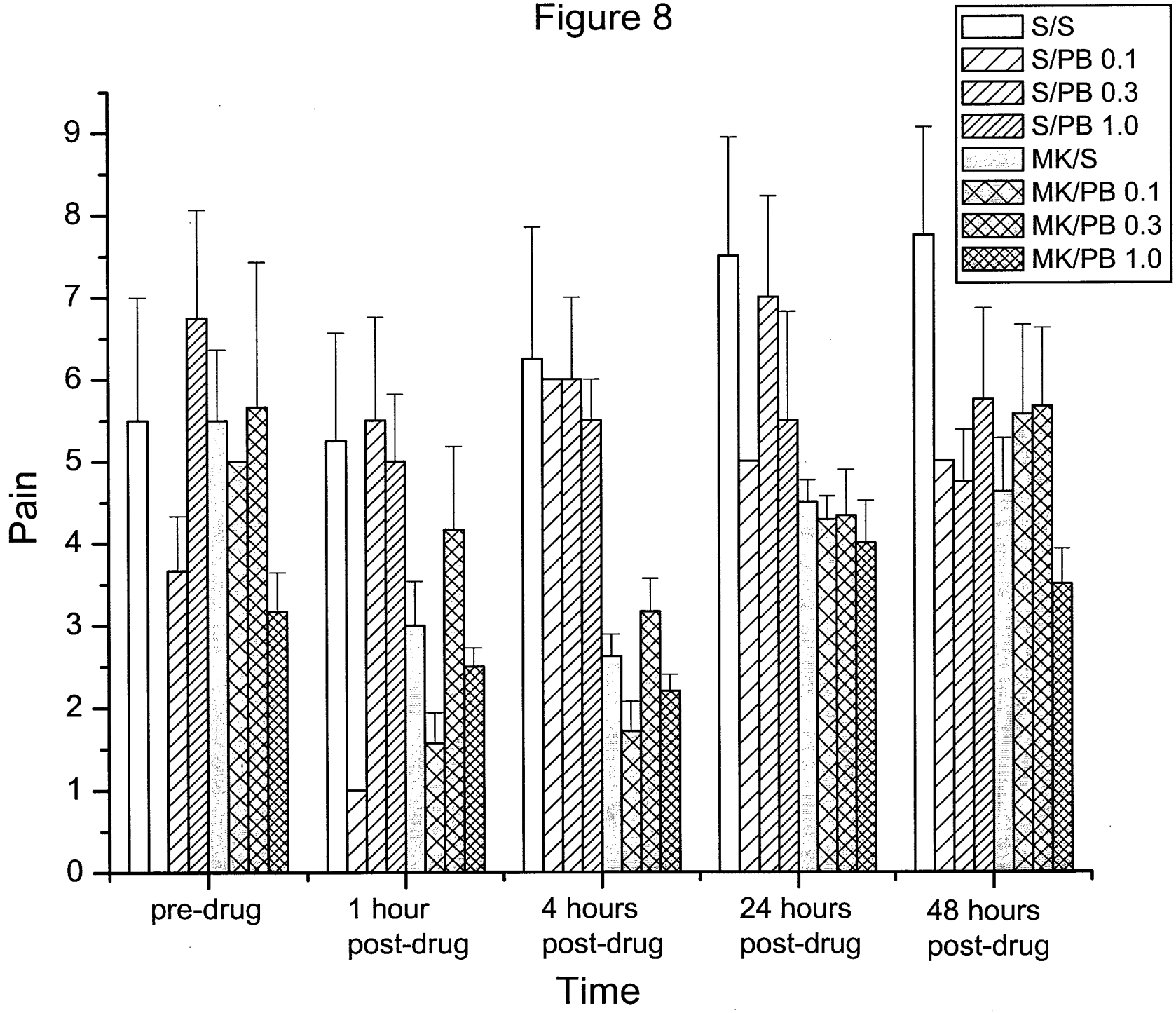

S/S
S/PB 0.1
S/PB 0.3
S/PB 1.0
MK/S
MK/PB 0.1
MK/PB 0.3
MK/PB 1.0
Sensorimotor
0
2
4
6
8
10
12
14
16
pre-drug
1 hour post-drug
4 hours post-drug
24 hours post-drug
48 hours post-drug
Time

Figure 7

Figure 8


S/S
S/PB 0.1
S/PB 0.3
S/PB 1.0
MK/S
MK/PB 0.1
MK/PB 0.3
MK/PB 1.0
Pain
0
1
2
3
4
5
6
7
8
9
pre-drug
1 hour post-drug
4 hours post-drug
24 hours post-drug
48 hours post-drug
Time

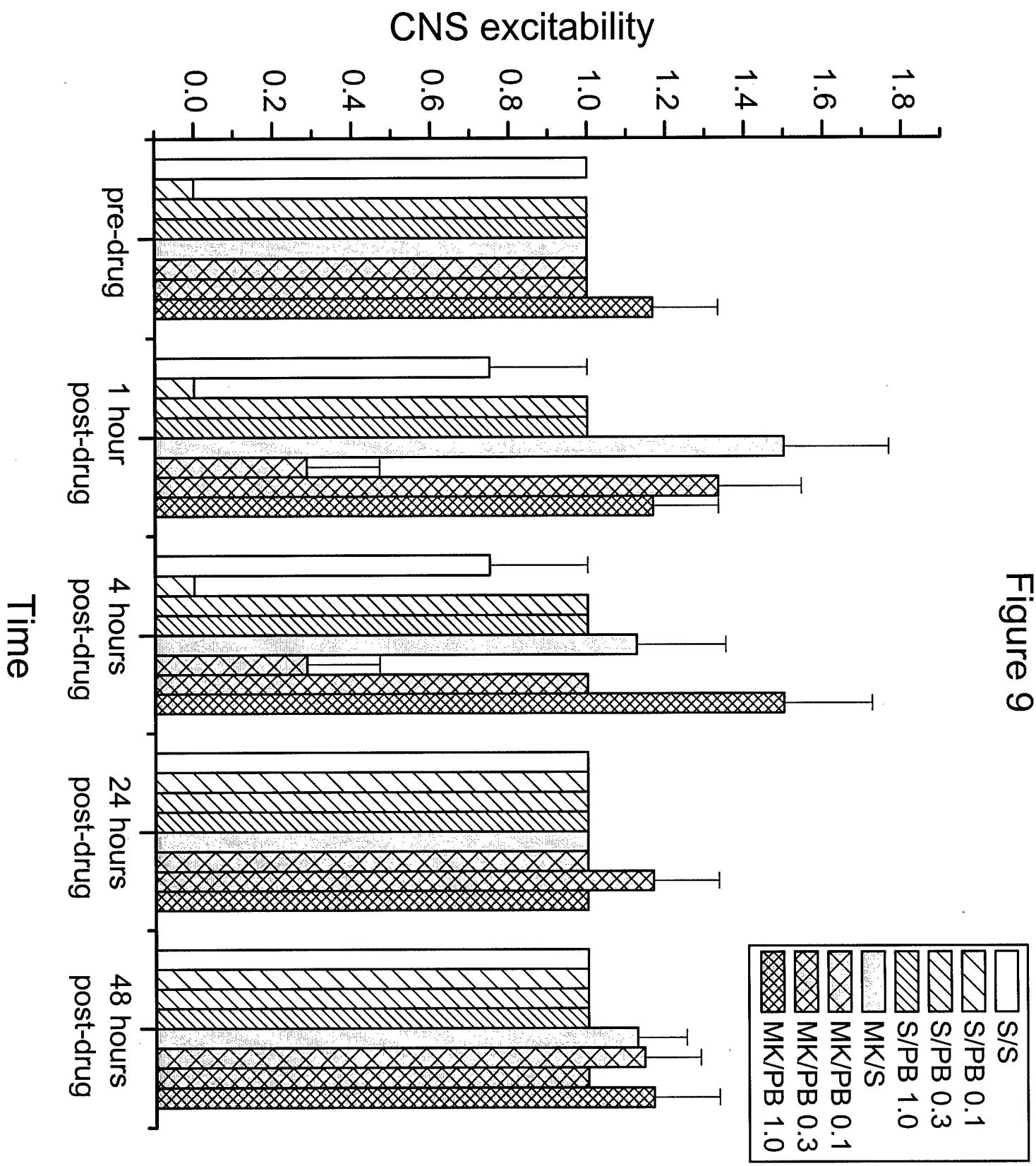

CNS excitability
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
pre-drug
1 hour post-drug
4 hours post-drug
24 hours post-drug
48 hours post-drug
Time
S/S
S/PB 0.1
S/PB 0.3
S/PB 1.0
MK/S
MK/PB 0.1
MK/PB 0.3
MK/PB 1.0

Figure 9

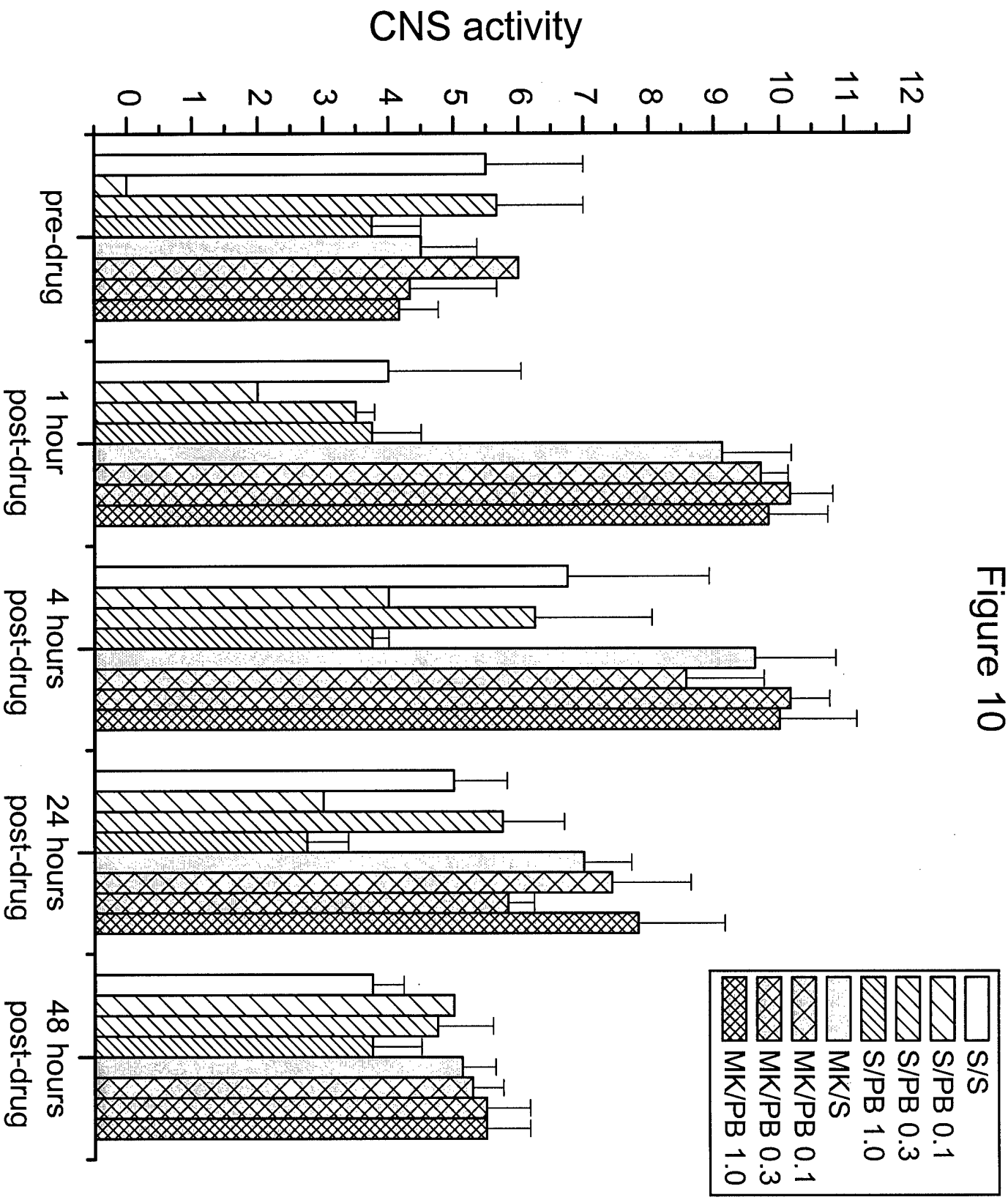

CNS activity
0
1
2
3
4
5
6
7
8
9
10
11
12
pre-drug
1 hour post-drug
4 hours post-drug
24 hours post-drug
48 hours post-drug
Time
S/S
S/PB 0.1
S/PB 0.3
S/PB 1.0
MK/S
MK/PB 0.1
MK/PB 0.3
MK/PB 1.0

Figure 10

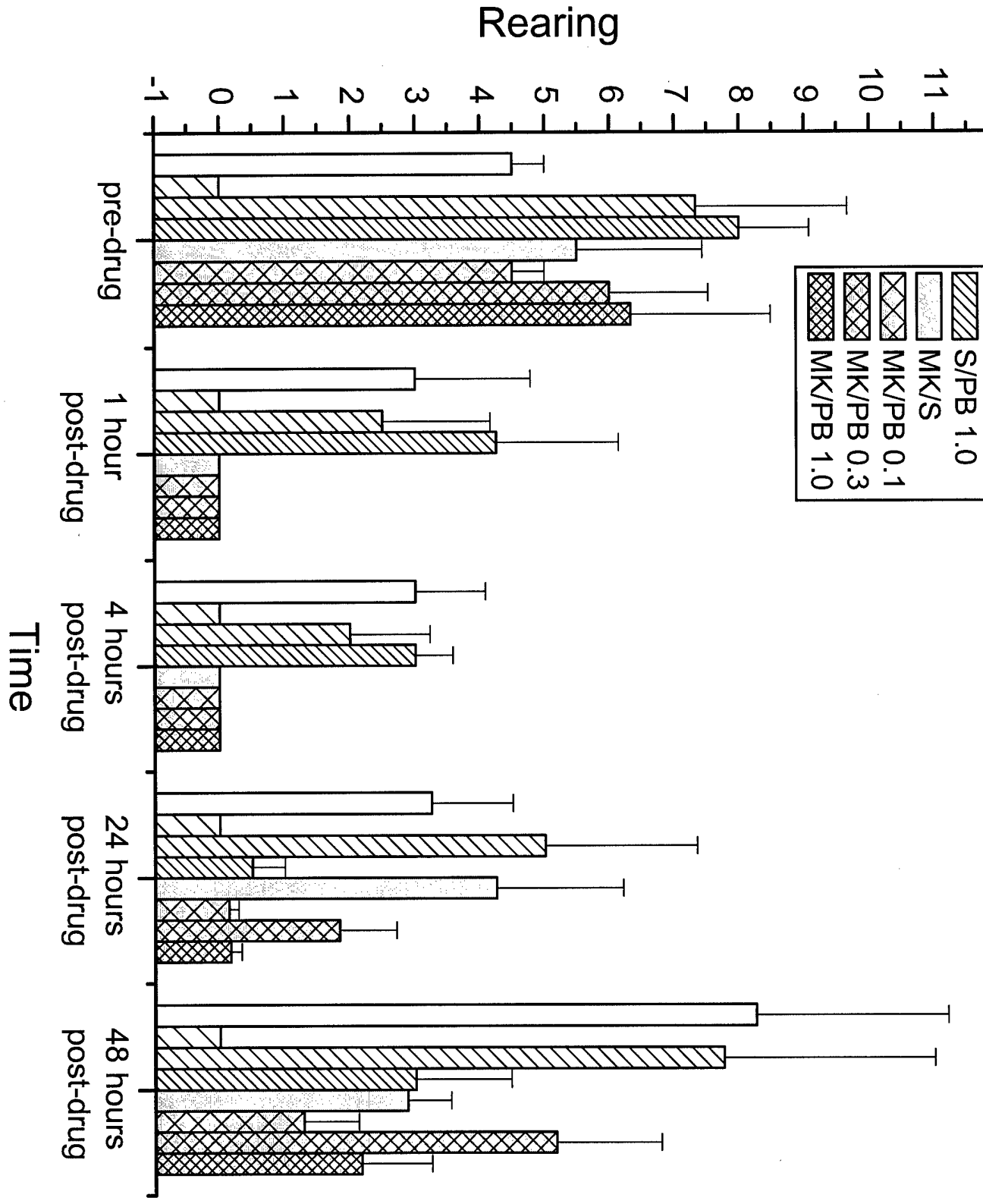

Rearing
-1
0
1
2
3
4
5
6
7
8
9
10
11
12
S/S
S/PB 0.1
S/PB 0.3
S/PB 1.0
MK/S
MK/PB 0.1
MK/PB 0.3
MK/PB 1.0
pre-drug
1 hour post-drug
4 hours post-drug
24 hours post-drug
48 hours post-drug
Time

Figure 11

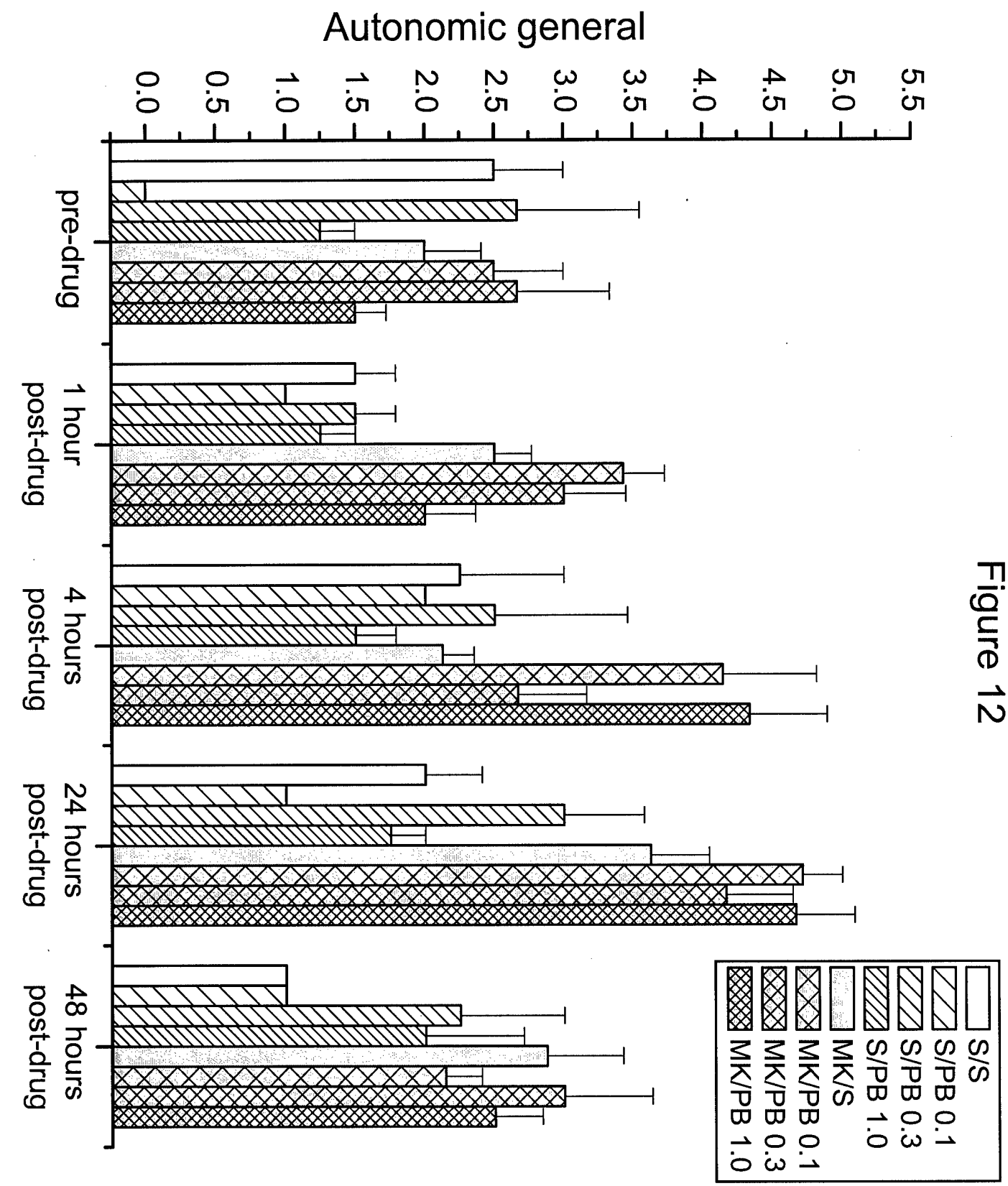

Autonomic general
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
pre-drug
1 hour post-drug
4 hours post-drug
24 hours post-drug
48 hours post-drug
Time
S/S
S/PB 0.1
S/PB 0.3
S/PB 1.0
MK/S
MK/PB 0.1
MK/PB 0.3
MK/PB 1.0

Figure 12

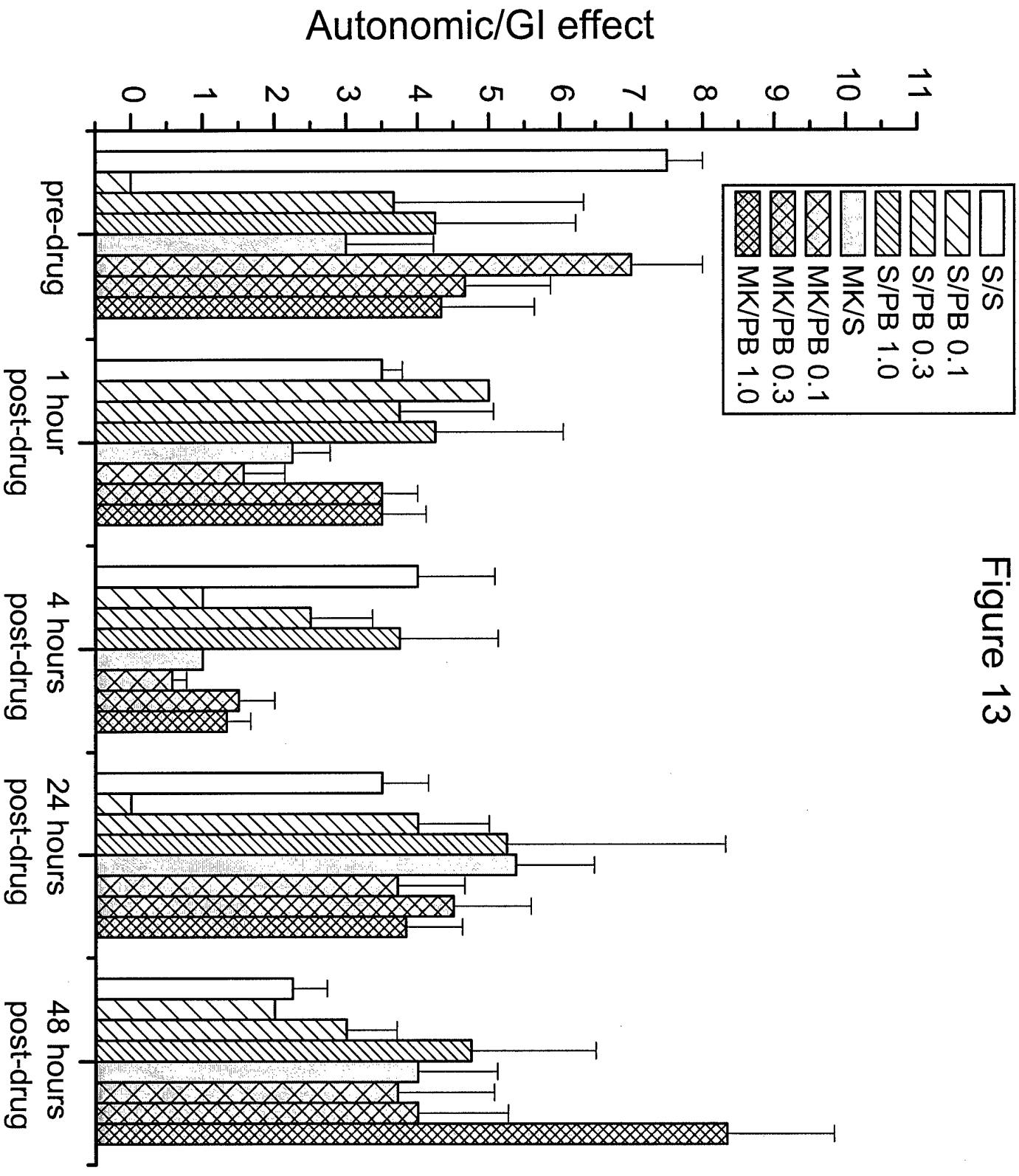

Autonomic/GI effect
0
1
2
3
4
5
6
7
8
9
10
11
S/S
S/PB 0.1
S/PB 0.3
S/PB 1.0
MK/S
MK/PB 0.1
MK/PB 0.3
MK/PB 1.0
pre-drug
1 hour post-drug
4 hours post-drug
24 hours post-drug
48 hours post-drug
Time

Figure 13

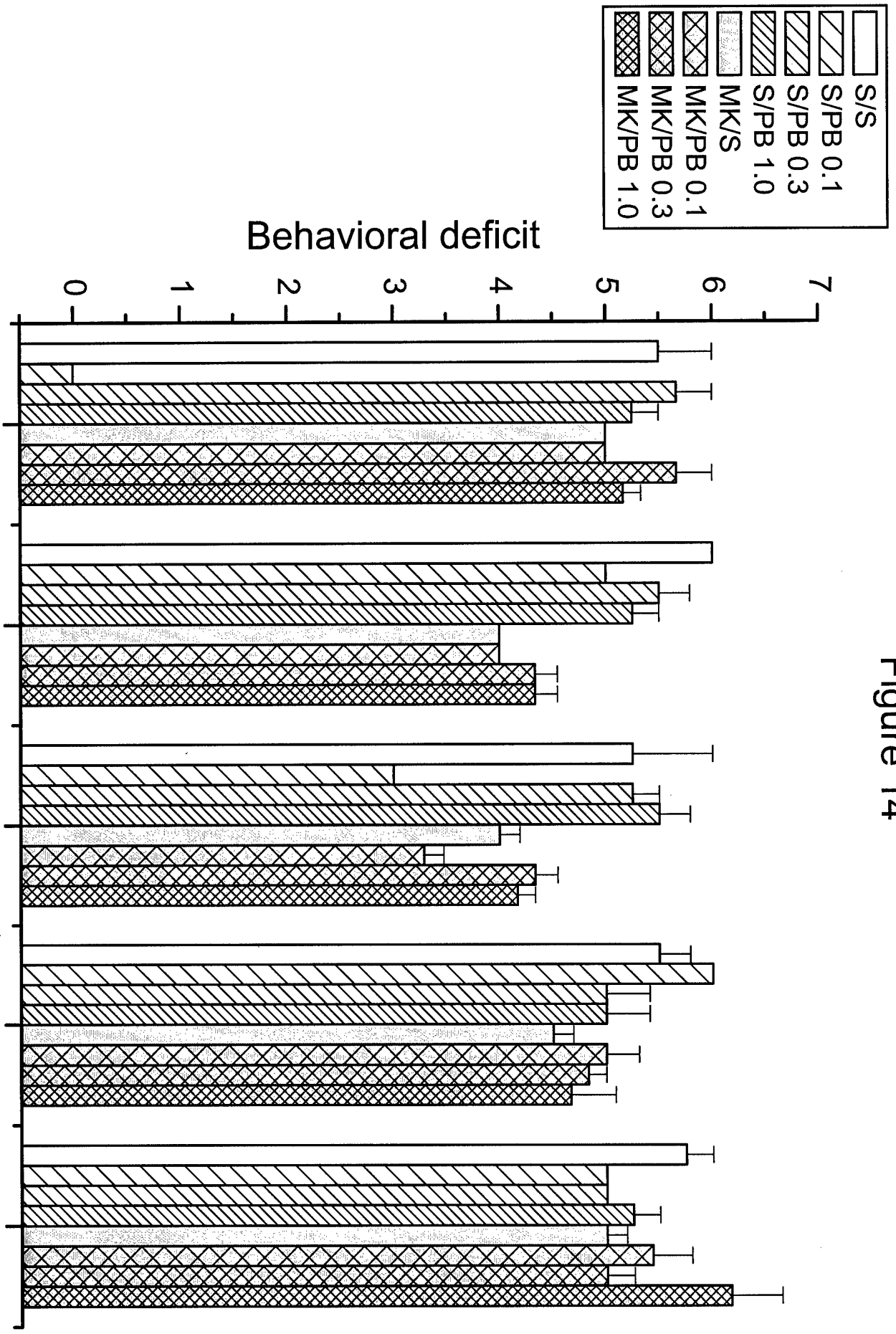
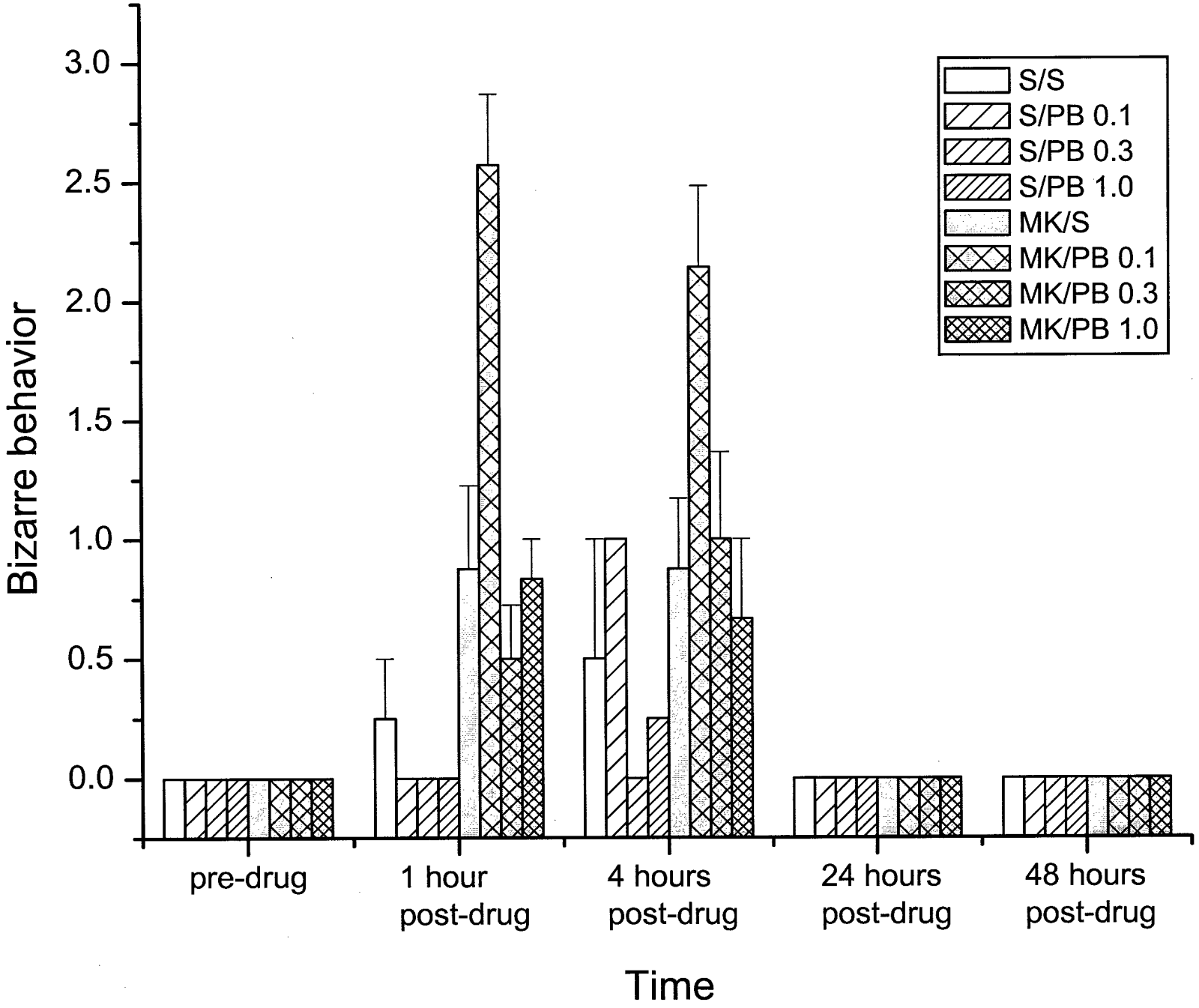

S/S
S/PB 0.1
S/PB 0.3
S/PB 1.0
MK/S
MK/PB 0.1
MK/PB 0.3
MK/PB 1.0
Behavioral deficit
0
1
2
3
4
5
6
7
pre-drug
1 hour post-drug
4 hours post-drug
24 hours post-drug
48 hours post-drug
Time

Figure 14

Figure 15


Bizarre behavior
3.0
2.5
2.0
1.5
1.0
0.5
0.0
S/S
S/PB 0.1
S/PB 0.3
S/PB 1.0
MK/S
MK/PB 0.1
MK/PB 0.3
MK/PB 1.0
pre-drug
1 hour post-drug
4 hours post-drug
24 hours post-drug
48 hours post-drug
Time

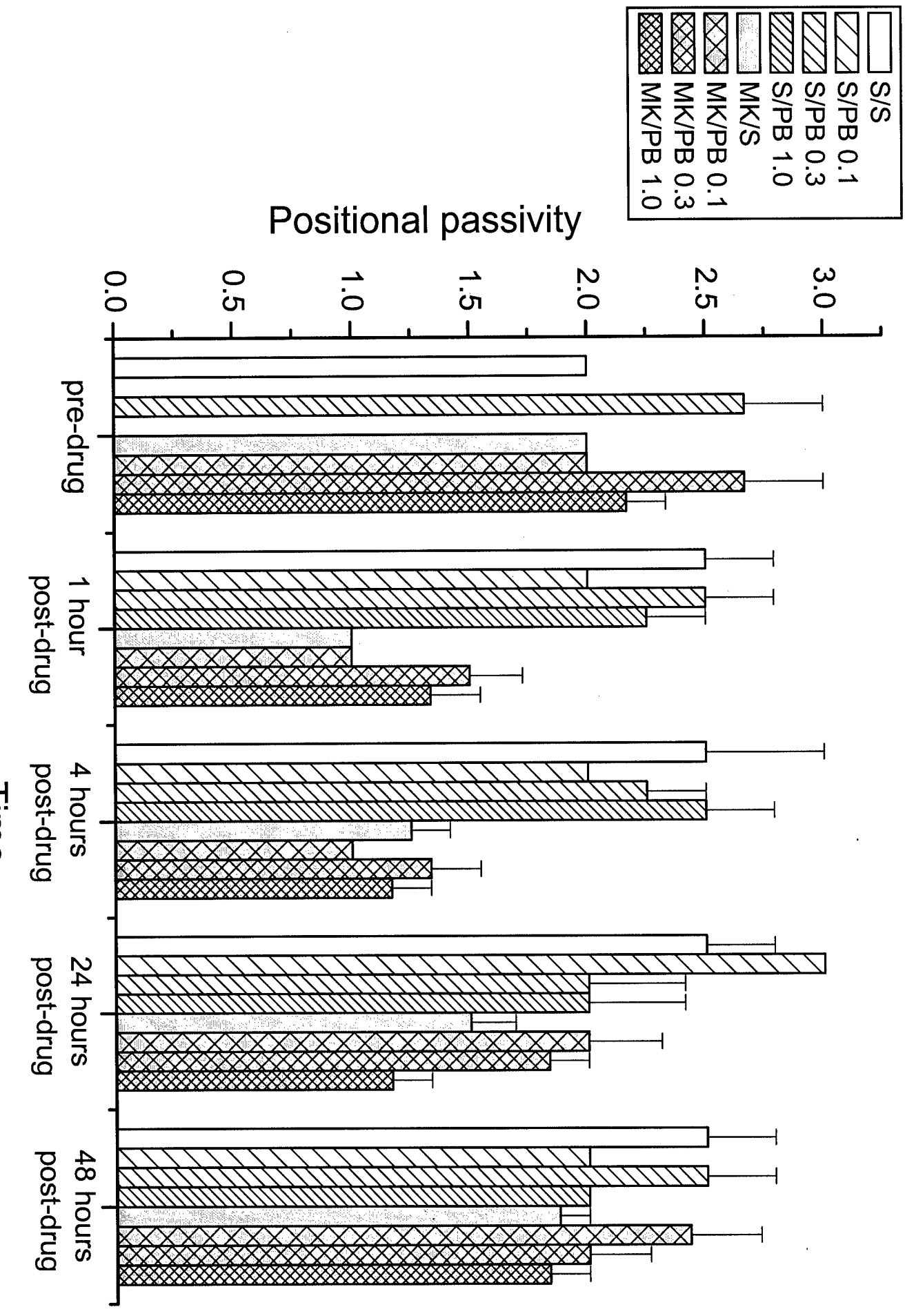

S/S
S/PB 0.1
S/PB 0.3
S/PB 1.0
MK/S
MK/PB 0.1
MK/PB 0.3
MK/PB 1.0
Positional passivity
0.0
0.5
1.0
1.5
2.0
2.5
3.0
pre-drug
1 hour post-drug
4 hours post-drug
24 hours post-drug
48 hours post-drug
Time

Figure 16

Figure 17

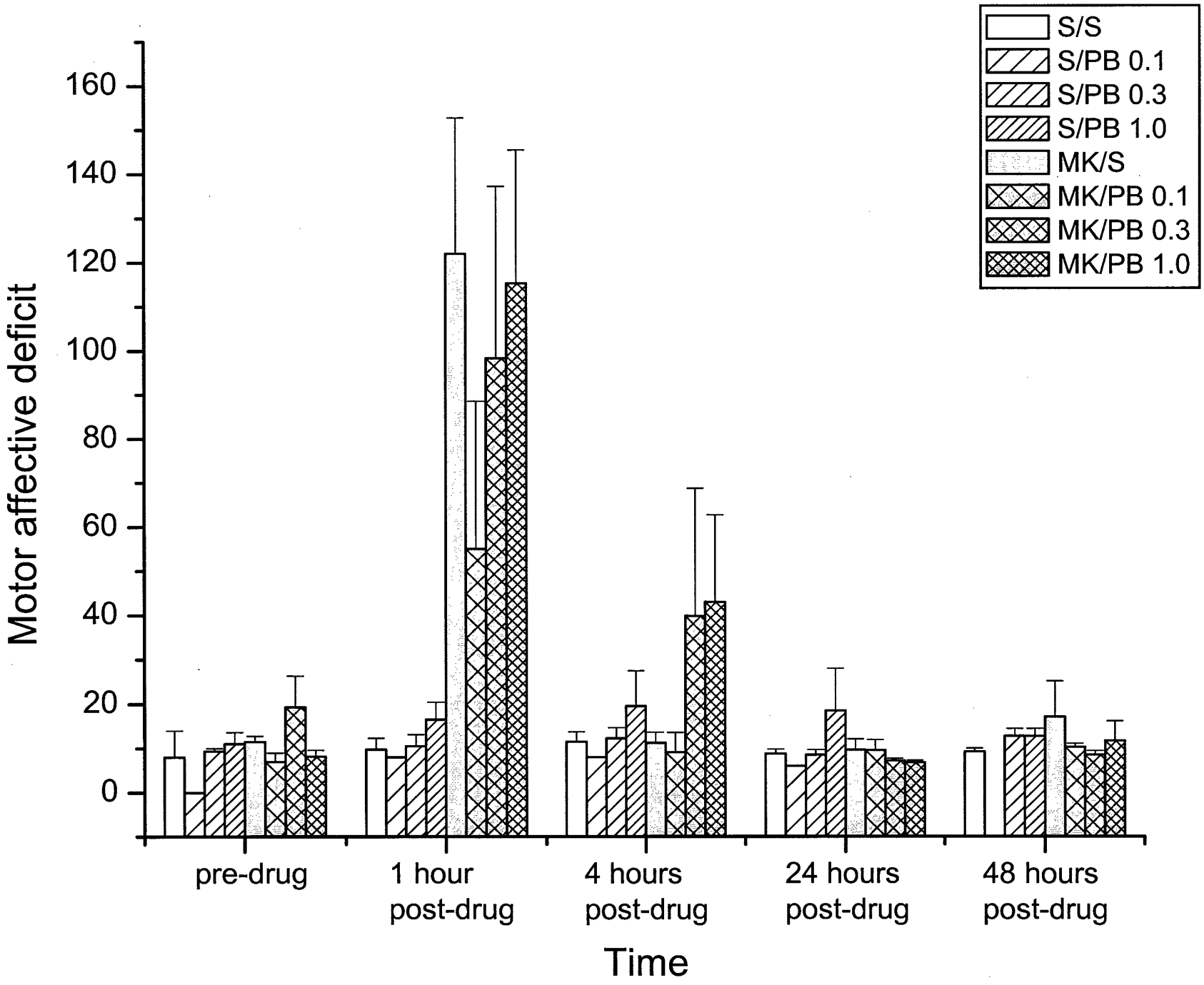

S/S
S/PB 0.1
S/PB 0.3
S/PB 1.0
MK/S
MK/PB 0.1
MK/PB 0.3
MK/PB 1.0
Motor affective deficit
160
140
120
100
80
60
40
20
0
pre-drug
1 hour post-drug
4 hours post-drug
24 hours post-drug
48 hours post-drug
Time

Figure 18

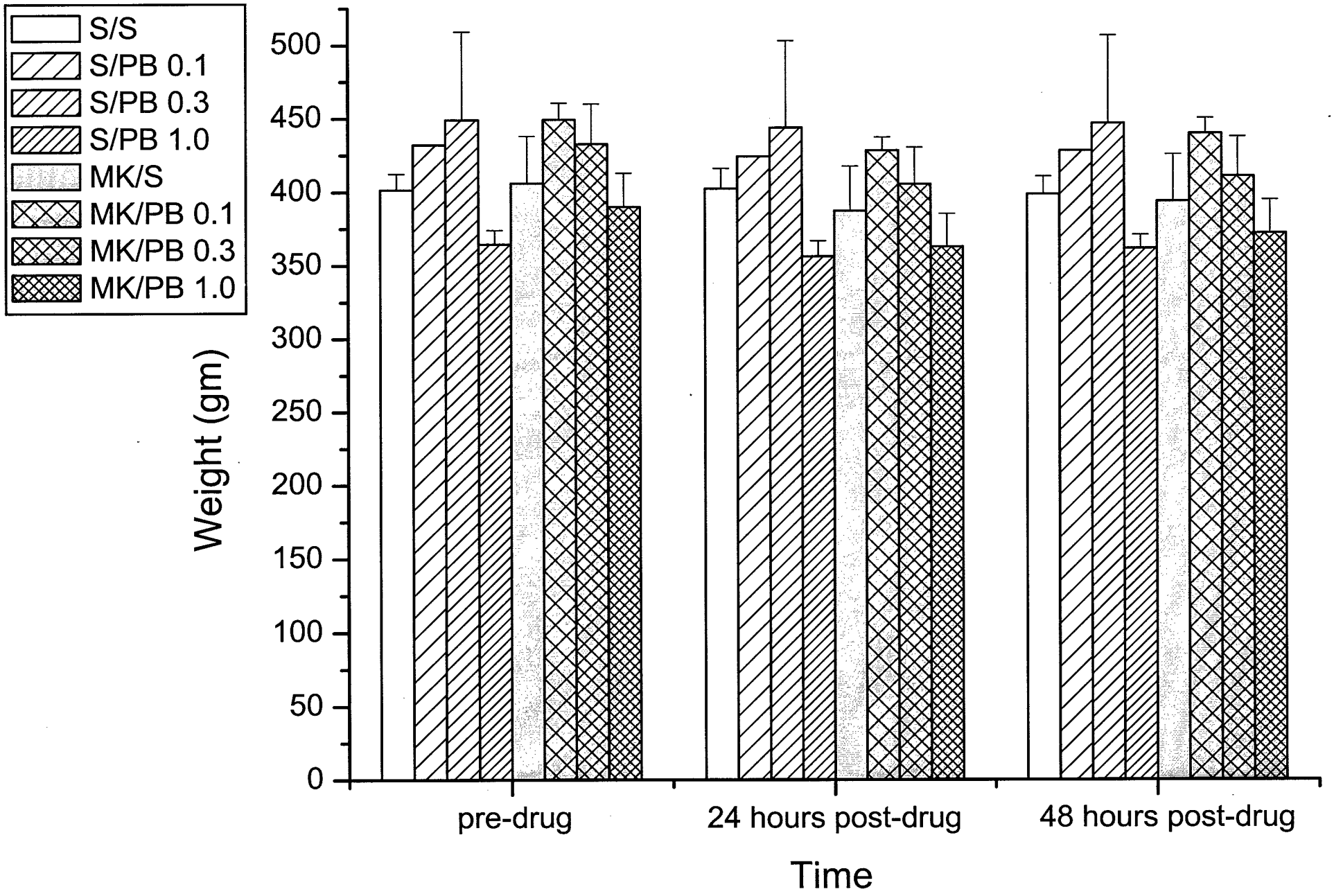

S/S
S/PB 0.1
S/PB 0.3
S/PB 1.0
MK/S
MK/PB 0.1
MK/PB 0.3
MK/PB 1.0
Weight (gm)
500
450
400
350
300
250
200
150
100
50
0
pre-drug
24 hours post-drug
48 hours post-drug
Time

Table 1

Experiment:		
Drug 1/route:		
Drug 2/route:		
Drug 3/route:		
Drug 4/route:		

Post-drug time:	
Date:	
Examiner init:	

Species:	Rat	Animal code:	1/Red	2/Green	3/Blue	4/Purple	5/Black	6/Orange	7/Grn/Blk	8/Bl/Blk
Strain:	Sprague-Dawley	Body weight (g):								
Sex:	female	Volume injected:								
Age:	retired breeders	Time admin:								
	measurement	Score								
1	Body posture	scale 1-10								
2	Bizarre behavior	scale 1-3, nature								
3	Tremors I	scale 1-5, nature								
4	Twitches	scale 1-5								
5	Convulsions I	clonic/tonic, scale								
6	Palpebral closure I	scale 1-4								
7	Exothalmos	present = 1								
8	Eye crustiness	present = 1								
9	Arousal	scale 1-6								
10	Convulsions II	clonic/tonic, scale								
11	Tremors II	scale 1-5, nature								
12	Time to 1st movement	seconds								
13	Palpebral closure II	scale 1-4								
14	Piloerection	present = 1								
15	Fur appearance	scale 1-3								
16	Rearing/3 min.	number/3 min								
17	Grooming/3 min.	number/3 min								
18	Tail elevation	scale 1-5								
19	Startle response	scale 1-3								
20	Provoked biting	scale 1-5								
21	Finger approach	scale 1-5								
22	Finger withdrawal	scale 1-5								
23	Touch-escape	scale 1-7								

		Animal code:	1/Red	2/Green	3/Blue	4/Purple	5/Black	6/Orange	7/Grn/Blk	8/Bl/Blk
24	Gait	scale 1-6								
25	Total gait incapacity	scale 1-4								
26	Limb rotation	scale 1-5								
27	Positional passivity	scale 1-5								
28	Visual placing	scale 1-5								
29	Grip strength	scale 1-5								
30	Hypothermia	present = 1								
31	Lacrimation	present = 1								
32	Toe-pinch	scale 1-5								
33	Pencil Grip	scale 1-3								
34	Extensor thrust	scale 1-4								
35	Diarrhea	present = 1								
36	Salivation	scale 1-5								
37	Tail pinch	scale 1-7								
38	Righting reflex	scale 1-4								
39	Catalepsy	scale 1-4								
40	Grasp irritability	scale 1-4								
41	Provoked freezing	scale 1-4								
42	Vocalizations	number of/3 min.								
43	Urination/defecation	number of/3 min.								
44	Death	present +								

Experiment:
Post-drug time:

Table 2

Functional domain:		1/Red	2/Green	3/Blue	4/Purple	5/Black	6/Orange	7/Grn/Blk	8/Bl/Blk
Neuromuscular	Tail elevation:								
	Gait score:								
	Gait incapacity:								
	Limb rotation:								
	Grip strength:								
	Body tone:								
	Pencil Grip:								
	Foot Splay:								
	Extensor thrust:								
	Total Score:								
Sensorimotor	Startle response:								
	Finger approach:								
	Finger withdrawal:								
	Touch-escape:								
	Visual placing:								
	Righting reflex:								
	Catalepsy:								
	Total Score:								
Pain	Toe-pinch:								
	Tail-pinch:								
	Total Score:								
CNS excitability	Convulsions II:								
	TremorsII:								
	Total Score:								
CNS activity	Body posture:								
	TremorsI:								
	Twitches:								
	Convulsions I:								
	Total Score:								

	Rearing:								
	Total Score:								
Autonomic/general	Palpebral closure I:								
	Exothalmos:								
	Eye crustiness:								
	Piloerection:								
	Hypothermia:								
	Lacrimation:								
	Light-pupil response:								
	Total Score:								
Autonomic/G.I.	Diarrhea:								
	Salivation								
	Defecation:								
	Total Score:								
Behavioral:	Bizarre behavior:								
	Palpebral closure II:								
	Fur appearance:								
	Grooming:								
	Provoked biting:								
	Positional passivity:								
	Total score:								
Motor affective:	Arousal:								
	Time to movement:								
	Grasp irritability:								
	Provoked freezing:								
	Vocalizations:								
	Total score:								

Key Research Accomplishments

1. The non-competitive NMDA antagonist MK-801 reduced bicuculline-sensitive IPSCs recorded in pyramidal cells in the PCC/RSC at doses as low as 3 μM, and this effect was statistically more prominent in an area vulnerable to NMDA antagonist-induced neurotoxicity (PCC/RSC) than in a less vulnerable area (parietal cortex). The competitive NMDA antagonist APV had a similar effect. These finding supports the hypotheses regarding the mechanisms underlying NMDA antagonist-induced neurotoxicity in the PCC/RSC.

2. The non-competitive NMDA antagonist MK-801 reduced NMDA receptor-mediated EPSCs recorded in interneurons in the PCC/RSC. This finding provides the first evidence that interneurons in this specific area are driven by NMDA receptor-mediated activity. This property could render the PCC/RSC vulnerable to hyperexcitability, leading to excitotoxicity in the presence of NMDA antagonists by the following mechanism: If, in the presence of an NMDA antagonist, inhibitory interneurons lose their excitatory drive, and if this results in excitatory pyramidal cells losing their GABAergic synapatic inhibitory drive, the net dis-inhibition of the excitatory cells could allow hyperexcitability to arise which, if unchecked, could lead to excitotoxicity. This finding directly supports the hypotheses regarding the mechanisms underlying NMDA antagonist-induced neurotoxicity in the PCC/RSC.

3. To confirm that bicuculline-sensitive synaptic GABAergic potentials recorded in pyramidal cells were not contaminated by other inputs, pyramidal cells were held at various potentials (e.g., to rule out contributions from NMDA receptors).

4. The muscarinic agonist pilocarpine and the AChEI physostigmine reduce bicuculline-sensitive IPSCs recorded in pyramidal cells in the PCC/RSC. At the doses used, this effect was additive with the effect of MK-801. These finding supports the hypothesis that cholinergic input (or an increase in cholinergic drive due to enzyme inhibition by an AChEI) can act directly in the PCC/RSC, and this effect could be an underlying mechanism by which pilocarpine enhances NMDA antagonist-induced neurotoxicity in vivo.

5. Ethanol, which possesses NMDA antagonist activity in some areas of the brain, can also block NMDA receptor-mediated EPSCs in pyramidal cells in the PCC/RSC. This effect is similar to the effect of ethanol in other brain areas (e.g., hippocampus and amygdala), but is the first reported finding of this effect in cortical neurons of the PCC/RSC. Thus suggests that ethanol could, in sufficient doses, produce neurotoxicity in the PCC/RSC similar to prototypical NMDA antagonist (but only if the other action actions of ethanol (e.g., action at GABAergic receptors) do not override the dis-inhibitory effect. In addition, ethanol is more effective against IPSCs in slices from young rats, compared to adults.

6. Age-related differences exist in the effect of the non-competitive NMDA antagonist MK-801 to reduced bicuculline-sensitive IPSCs recorded in pyramidal cells in the PCC/RSC. The time required to reduce the peak IPSC amplitude to 50 % is shorter in slices from adult rats than in slices from young rats. This suggests that MK-801 may have a faster onset of action in adults, which could be a mechanism by which adults are more sensitive to the neurotoxic effects of NMDA antagonists.

7. MK-801 produced neurodegeneration, as measured using the Fluoro-Jade B stain, at doses (0.3 mg/kg) lower than previously reported. Neither pyridostigmine bromide nor physostigmine produce Fluoro-Jade-B-detectable neurodegeneration at doses of no or low toxicity in vivo.

8. MK-801, pyridostigmine bromide and physostigmine produce behavioral changes that can be consistently measured using a functional observational battery test. Repeated measures of FOB can provide a measure of functional recovery (or lack thereof) from exposure to NMDA antagonists plus-or-minus AChEIs. Thus, behavioral changes can be correlated with subsequent findings in histopathological studies in each animal.

Reportable Outcomes:

Abstracts:*

1. EFFECT OF ETHANOL ON NMDA RECEPTOR-MEDIATED EPSCs OF PYRAMIDAL CELLS IN THE POST CINGULATE GYRUS OF JUVENILE AND ADULT RATS. Q. Li., W.A., Wilson., and H.S. Swartzwelder. Departments of Pharmacology and Cancer Biology, Psychiatry, and Psychology, Duke University Medical Center. Neurology Research Laboratory, VA Medical Center, Durham, NC 27705 USA (Poster presented at the Resarch Alcohol Assn., Montreal, Quebec, July 2001).

2. Li, et al., Soc. Neurosci. Abst. 2001a

3. Li, et al., Soc. Neurosci. Abst. 2001b

Manuscripts:*

1. Li, Qiang, Clark, S., Wilson, W.A., Lewis, D.V., MK-801, a NMDA Receptor Antagonist, Modulates the Inhibitory Postsynaptic Currents (IPSCs) in Pyramidal Neurons in the Rat Cingulate Gyrus, Journal of Neuroscience (*accepted pending revisions on July 2001*)

2. Li, Qiang, Wilson, W.A., H. Scott Swartzwelder, Differential effect of ethanol on NMDA receptor-mediated EPSCs in pyramidal cells in the posterior cingulate cortex of juvenile and adult rats. *Journal of Neurophysiology* (accepted, in press)

*Copies of the abstracts and the manuscript are provided in the Appendicies.

Conclusions:

The mechanisms by which the NMDA receptor/channel antagonist MK-801 causes neuronal damage in the cingulate gyrus and retrosplenial cortex have been investigated using intracellular patch-clamp electrophysiological recordings. We have used rat barin slices that include PCC/RSC and parietal cortex to test the hypothesis that MK-801 produces neurotoxicity by disrupting GABAergic inhibition, and that this disruption is greater in the cingulate cortex than other cortical areas. We have also obtained evidence that muscarinic agonists and AChEI's can produce a similar effect in the PCC/RSC. This finding could explain the mechanism by which pilocarpine can exacerbate NMDA antagonist-induced neurotoxicity – and may predict that AChEIs will also cause synergistic neurotoxic interactions. Expanded behavioral monitoring methods have been developed, and these will be used to correlate histopathological findings with behavioral changes following exposure to NMDA antagonists and AChEIs.

Our electrophysiological data has been the first (and, at present, remains the only) study to find that excitatory drive from excitatory pyramidal cells to inhibitory interneurons can be modulated by MK-801 in the PCC/RSC to a greater extent than in cortical areas less vulnerable to NMDA antagonist-induced neurotoxicity. Our studies are also unique in the methods used to explore the mechanisms by which NMDA antagonists and cholinergic agents interact to produce enhanced neurotoxicity. Given the risk of co-exposures to these agents – and *the potential need* for such co-exposures in certain military and civilian settings, it is important to identify and understand these neurotoxic mechanisms.

References

(Note: This list of references includes citations for the Overview section, as well as for both Part I and Part II of the Body. In addition to the references listed below, lists of references relevant to each study are included at the end of every manuscript attached in the Appendices.)

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Supplement to:
May 2001
vol 25 • no 5

www.alcoholism-cer.com

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13 SYNERGISTIC EFFECTS OF ETHANOL WITHDRAWAL AND HIV-1 *TAT* PROTEIN ON HIPPOCAMPAL DAMAGE IN RAT.

M.A. Prendergast and A. Nath. University of Kentucky, Department of Psychology and Department of Neurology. Lexington, KY 40546-0236.

Long-term ethanol exposure produces adaptive responses in multiple neurotransmitter systems that may contribute to the development of CNS excitability and neurodegeneration *in situ* and/or during ethanol withdrawal. Some of these adaptive responses, such as increased expression and/or function of *N*-methyl-D-aspartate type glutamate receptors (NMDAr), may render individuals more susceptible to neuronal insult produced by excitotoxins such as the HIV-1 transcription factor *TAT*, a protein associated with the development of AIDS-dementia. The present studies were designed to test the hypothesis that withdrawal from chronic ethanol exposure would sensitize the rat hippocampus to neurodegenerative effects of *TAT* , an effect postulated to be associated with increased function of the NMDAr. Organotypic slice cultures of male rat hippocampus were exposed to ethanol (100mM) for 10 days and withdrawn for 24 hours in the presence of 30 μM MK-801, 100-500 nM tetrodotoxin, or 10-500 nM *TAT*. Hippocampal damage was quantified via microscopy of the non-vital fluorescent marker propidium iodide. Withdrawal from ethanol exposure produced significant damage in the CA1 pyramidal cell layer , but not in other hippocampal regions. This was associated with marked increases in CA1 pyramidal cell binding of the fluorescent Ca^{2+} indicator, calcium orange. Damage and Ca^{2+} binding was reduced by exposure to MK-801 or tetrodotoxin during the withdrawal. *Tat*, at 500 nM, did not alter neuronal viability in any region when given alone. However, *Tat* exposure during ethanol withdrawal produced a marked increase in CA1 neurodegeneration. These data indicate that withdrawal from chronic ethanol exposure produced hippocampal network excitotoxicity that was mediated by NMDAr and voltage-dependent Na^+ channel function. Further, a synergistic effect on neurodegeneration was observed between prior ethanol exposure and exposure to HIV-1 *TAT* protein. These data suggest, then, that chronic ethanol intake may be related to increased risk for the development of AIDS-related dementia.

Supported by The National Institute on Alcohol Abuse and Alcoholism (MAP) and The National Institute of Neurological Disorders and Stroke (AN).

14 CORRELATION BETWEEN ETHANOL AND IFENPRODIL EFFECTS ON NMDA RECEPTOR DEPENDENT LONG TERM DEPRESSION.

C Miao, MJ Lippmann and RA Morrisett. Waggoner Ctr Addiction Res, Univ Texas, Austin, TX 78712.

It is well accepted that repetitive low frequency stimulation (LFS) of the Schaffer Collateral-CA1 synapse elicits long-term depression of hippocampal synaptic transmission (LTD). This form of synaptic plasticity is mediated by NMDA receptor activation, low level calcium influx and activation of protein phosphatases 1/2A which subsequently decreases AMPA channel function. Here we test the effect of ethanol and different NMDA receptor antagonists on the induction of NMDA-LTD.

Extracellular recordings were made from in area CA1 of hippocampal slices prepared using standard methods. We observed a strong and long-lasting depression of the pEPSP slope (-26% ±3%, n=14 slices from 8 rats following a prolonged LFS (1 Hz-7 min). When the LFS was delivered in the presence of D-APV, a potent and specific antagonist of NMDA receptor, minimal changes in the pEPSP slope were observed (-7±4%, 6 slices from 4 rats), indicating the NMDA receptor dependence of the induction of LTD. Interestingly, in the presence of 75 mM ethanol, there was no change in the induction of LTD versus the control condition (-29±5%, 20 slices from 9 rats). Further, when the low frequency stimulation is delivered in the presence of ifenprodil (10 μM), a NR2B antagonist, no blockade of the induction of LTD was observed (-38±4% pEPSP slope, 13 slices from 7 rats).

These data suggest that ethanol has little effect on the induction of the NMDA dependent LTD and that the NMDAR-2B subtype does not contribute to this form of synaptic plasticity. Supported by TCADA and NIAAA 11845 to RAM.

15 ANTAGONISTS FOR POLYAMINE-SITES ON THE NMDA RECEPTOR INHIBIT ALCOHOL WITHDRAWAL-INDUCED CELL DEATH IN ORGANOTYPIC RAT HIPPOCAMPAL SLICE CULTURES.

DA Gibson, MA Prendergast, JB Blanchard, BR Harris, J Littleton. University of Kentucky, Department of Pharmacology, Lexington KY.

Chronic alcohol intake produces many adaptive responses in the brain, including increases in *N*-methyl-D-aspartate receptor (NMDAR) activity. This increase in receptor activity has been implicated in the neurotoxicity produced by alcohol withdrawal in many studies. Although the mechanism for this increase is largely unknown, recent reports have shown increased production of polyamines following chronic ethanol exposure. This is of great interest since the polyamines spermidine and spermine have been shown to potentiate the actions of glutamate on the NMDAR. We therefore hypothesized that blocking these potentiating actions of polyamines at specific sites on the NMDAR during alcohol withdrawal would result in decreased neurotoxicity. To test this hypothesis, we utilized an organotypic hippocampal slice culture preparation in which cell death was assessed following removal of the slice cultures from a 10 day exposure to media that contained ethanol. This protocol produces NMDA receptor-mediated toxicity in the CA1 hippocampal region consistent with data reported from *in vivo* studies. Inhibiting the actions of polyamines on the NMDAR during ethanol withdrawal was accomplished using the putative polyamine-site antagonists ifenprodil, arcaine, and agmatine. Results obtained in these experiments showed that all three compounds were effective at reducing ethanol withdrawal-induced cell death in this preparation. Ifenprodil and agmatine reduced toxicity in the CA1 region of the hippocampus in a concentration-dependent manner, with a maximal reduction of nearly 100% for ifenprodil at 100μM and greater than 85% for agmatine at a concentration of 1μM. Arcaine was the least effective of the compounds tested, producing a maximal decrease in ethanol withdrawal-induced cell death of 52% at a concentration of 1μM. These results suggest that inhibition of the NMDAR at the polyamine-sites may be useful for both the treatment of alcohol withdrawal-induced neurotoxicity and for studying the role of polyamines in changes in NMDAR activity following chronic alcohol exposure.

16 EFFECT OF ETHANOL ON NMDA RECEPTOR-MEDIATED EPSCs OF PYRAMIDAL CELLS IN THE POSTERIOR CINGULATE GYRUS OF JUVENILE AND ADULT RATS.

Q. Li., W.A., Wilson., and H.S. Swartzwelder. Departments of Pharmacology and Cancer Biology, Psychiatry, and Psychology, Duke University Medical Center. Neurology Research Laboratory, VA Medical Center, Durham, NC 27705 USA

The inhibitory effect of EtOH on NMDA receptor-mediated synaptic transmission was investigated in neocortical slices using whole cell patch clamp recording. In the presence of DNQX (20μM) and BMI (40μM), NMDA receptor-mediated EPSCs were isolated from pyramidal cells of the post cingulate gyrus in brain slices from 1 and 3 month old rats. In slices from juvenile rats, 5, 10, 30 and 60mM EtOH reduced the mean amplitude of NMDA mediated EPSCs by 11.99, 22.84, 35.75 and 46.23%, respectively (n=17). However, the same concentrations of EtOH inhibited the mean amplitude of EPSCs by only 4.23, 8.43, 15.14 and 31.22%, in slices from adult rats compared to controls (n=11). In addition, 60mM EtOH caused a significant decrease in decay times of EPSCs in juvenile rats compared to adult rats. The results indicate that EtOH reduces NMDA receptor-mediated synaptic transmission more powerfully in juvenile rats compared to adults. *This work was supported by NIAAA Grant # 11088 to HSS, and 12478 to HSS & WAW, and by VA Senior Research Career Scientist Awards to HSS & WAW.*

17 IN-VITRO COMPARISON OF THE EFFECTS OF ETHANOL ON NMDA-GATED CURRENTS FROM BRAIN AREAS WITH DIFFERING IN-VIVO SENSITIVITY TO ETHANOL.

H.E. Criswell, B. L.Griffith and G.R. Breese, Bowles center for alcohol studies, U.N.C. Chapel Hill, Chapel Hill NC 27599.

Both systemic and locally applied ethanol decrease the rate enhancing effects of NMDA on neurons in the medial septum or substantia nigra reticulata (SNR) but have no effect on neurons from the lateral septum (LS); Simson et al., J.P.E.T.,1991, 257:225-231). Because the in-vivo studies did not distinguish between pre- and post-synaptic actions of ethanol, the present work examined the effect of ethanol on NMDA-gated currents in neurons acutely isolated from the SNR or LS of 15 to 20 day old rats. The lack of presynapitc inputs to these acutely isolated neurons limits the action of ethanol to post-synaptic sites.

Whole-cell voltage clamp was used to compare currents mediated by brief applications of NMDA or NMDA plus varying concentrations of ethanol. Ethanol produced a concentration dependent decrease in the current gated by 100 μM NMDA. Percent inhibition (±SEM) of NMDA-gated currents by 25 mM ethanol was 11.1± 3.05 vs. 11.5± 2.2 for the LS and SNR respectively. Inhibition by 50 mM ethanol was 18.7±4.0 vs. 19.4± 2.0 and at 100 mM percent inhibition was 45.7±6.0 and 34.9±1.9. Two way ANOVA showed a significant effect of dose ($P<0.01$) but no difference between brain sites ($P>0.1$). Thus, the differential effect of ethanol observed in vivo between neurons in the LS and SNR does not appear to result from differential effects of ethanol at the postsynaptic NMDA receptor. Supported by NIAAA.

18 EFFECT OF TRANSMEMBRANE DOMAIN THREE MUTATIONS ON THE ETHANOL SENSITIVITY OF NMDA RECEPTORS.

K.M. Ronald, T. Blevins, and J.J. Woodward. Dept. of Pharmacology, Medical College of Virginia Campus at Virginia Commonwealth University, Richmond, VA 23298.

Ethanol has been shown to significantly inhibit NMDA receptors but the mechanism of action remains elusive. Alcohols are hypothesized to act on the NMDA receptor by partitioning into an area defined by specific amino acids. To determine if ethanol interacts with amino acids in the transmembrane (TM) domains, specific amino acids have been changed to alanine, expressed in *Xenopus* oocytes, and tested for sensitivity to ethanol. The NR1 TM3 mutant F639A significantly decreased ethanol's inhibition of the receptor when co-expressed with NR2A, NR2B, or NR2C subunits. This effect was not due to a non-specific disruption of receptor structure because alteration of the neighboring amino acid M641 did not affect the ethanol sensitivity of the receptor. In addition, alteration of F639 to the larger amino acid tryptophan slightly enhanced ethanol's inhibitory effects. NR1(F639A) did not alter the potency or efficacy of glutamate when co-expressed with NR2A subunits. However, this mutant showed enhanced glycine sensitivity when combined with NR2A or NR2B subunits. NR1(F639A)/2A receptors were activated to a greater extent by the partial agonist (+)-HA-966 than wild-type receptors suggesting that this mutant may enhance glycine's ability to open the channel. Taken together, these results suggest that F639 may represent an important site that regulates the overall ethanol sensitivity of NMDA receptors. The work was supported by AA09986 (JJW), KO2-AA00238 (JJW), and T32-DA07027 (KMR).

419 352-6461

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July 18, 2001

Dr. Qiang Li
Neurology Research, Room 24, Bldg. 16, VAMC
508 Fulton Street
Durham, NC 27705 USA

RE: JN1851-01

Dear Dr. Li:

The reviews of your paper, "NMDA Receptor Antagonists Disinhibit Rat Posterior Cingulate and Retrosplenial Cortices: a Potential Mechanism of Neurotoxicity," have been received by Dr. Barry W. Connors, your Reviewing Editor, and by me. As you will see from the reviews, the reviewers found considerable merit in your work but also raised substantial concerns about the paper. Consequently, to be acceptable for publication, your paper must be adequately revised along the lines suggested by the reviewers and then submitted for re-review. The reviewers' comments are enclosed.

The concerns of the reviewers and suggestions for improvement of the manuscript are clearly set out in the reviews. It is critical that you address these concerns effectively in your revised manuscript and also in a cover letter that provides point-by-point responses to the reviewers' substantive comments. A final decision about the manuscript will be made once the Reviewing Editor and I are able to evaluate the re-review along with the material you have provided.

Please return your revised paper as soon as possible. As a matter of policy, manuscripts returned after three months will be treated as new submissions.

When the necessary revisions have been made, please send five copies of the revised manuscript (one with an original set of illustrations, three with reviewer-quality illustrations) *plus one extra photocopied set of illustrations only* to **Managing Editor, *The Journal of Neuroscience*, Society for Neuroscience, 11 Dupont Circle, NW, Suite 500, Washington, DC 20036.** One copy of the manuscript and illustrations should be sent to me. Dr. Connors will receive a copy of the manuscript from the Central Office. Please be sure that the Managing Editor and I receive copies of the cover letter explaining your revisions. *Please submit the revised manuscript and cover letter on disk to expedite production.* Specific instructions are attached. If you are submitting digital art, complete the Digital Art Submission Checklist included in this packet and return it with your revised manuscript. Please visit http://cjs.cadmus.com/da for detailed instructions on how to prepare digital art and to ensure that you have prepared your digital art files correctly.

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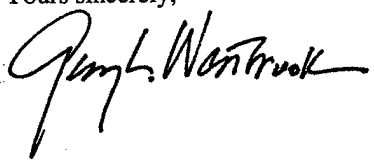

Gary L. Westbrook, M.D.
Senior Editor

GLW/rf
Enclosure

JN 1851-01 Reviewer #1
Comments to Authors:

This paper examines the effect of NMDA receptor antagonists on IPSCs in rat neocortex. The hypothesis is that disinhibition via blockade of NMDA excitation of interneurons could be a potential mechanism for neurotoxicity. The authors have used a slice preparation to test if there are region specific differences in NMDA antagonist induced disinhibition. They conclude that NMDA receptors regulate the activity of inhibitory interneurons and GABA release, in a region specific manner. These results are interesting and could be important for understanding how NMDA antagonists produce neurotoxicity.

There are several problems with the paper as it currently stands. The first deals with unequivocal demonstration that IPSCs were indeed being studied while another is the sample size for parietal cortex. A final issue is a better review of relevant literature.

Recordings were obtained at +30 mV. Under the recording conditions employed EPSCs and IPSCs would both be outgoing and difficult to distinguish. MK-801 could block NMDA mediated EPSCs resulting in a decrease in activity. The remaining activity would be IPSCs and therefore BMI sensitive. AMPA mediated EPSCs might not be seen at positive potentials since they can show marked rectification. Fig. 2 should include recordings at -70 mV in the presence of BMI to assess the presence of EPSCs. If such activity is present then the effects of MK-801 need to be assessed at +30 in the presence of BMI to see how EPSCs are affected.

The results in Fig. 2 D are surprising. Neocortical cells usually respond to stimulation with EPSCs and IPSCs which overlap temporally. The reversal potential measure in this cell suggests a lack of an EPSC contribution. The number of times this was observed and the average reversal potential need to be specified. Actual data points should be included in Fig. 2E and the time of the amplitude measurement given.

It is stated that pyramidal cells were recorded primarily in layers II to V of neocortex (pg.10). This is somewhat vague; were layer VI cells recorded? In fact, the distribution of cells needs to be specified and the effects of MK-801 on different layers examined. Inhibition in upper cortical layers has been suggested to differ from deeper layers.

The number of cells in parietal cortex is small and the laminar distribution not stated. Given the variability seen in PCC/RSC (23% showing

a slight or not significant effect), seven cells is too small a sample to basis a major conclusion (area specific vulnerability).

Previous investigations of NMDA receptor antagonists on IPSC frequency (J. Neurophysiol 1996; 75:1573) did not show a strong effect. This could be used as evidence for a region specific effect but should be cited in any case.

There are several paper showing NMDA receptors on interneurons in neocortex (Br Res 1998, 780:166) although this has not been true in hippocampus (J Physiol 1993, 462:373.). This is an important issue for the proposed model and should be discussed.

The use of "etc" on pg. 4 is not very scholarly. It is also not clear (pg. 4,4 lines up from bottom) what the "core excitatory/inhibitory network" is.

Pg. 6, HEPES not HEEPS
Pg. 7 gigohm not GM. The series resistance values should be given as well as what was a significant change.

JN 1851-01 Reviewer #2
Comments to authors:

This manuscript describes experiments that are intended to address a very interesting and important paradox: NMDA receptor antagonists actually do not prevent seizures and excitotoxicity but can exacerbate them. This issue first arose because of the clinical failure of NMDA receptor antagonists for seizures. In addition, rat studies showed that the NMDA receptor antagonist MK-801 led to neurodegeneration. The authors have tested a very straightforward hypothesis for this apparent paradox and provide evidence for it. The hypothesis is that inhibitory neurons, particularly in the areas of the cortex that are vulnerable to NMDA antagonists, are strongly activated by NMDA receptors. Thus, it is hypothesized that by blocking their activation, inhibitory neurons become less effective and disinhibition results.

The test of this hypothesis is indirect, but it is reasonable. The authors use recordings of miniature, spontaneous, and evoked IPSCs in pyramidal neurons in two areas of cortex, one that is vulnerable to MK801 and one that is not. The NMDA antagonists MK-801 and APV are used to show that IPSCs decrease, but the analysis of miniatures does not suggest a pre- or postsynaptic locus of the effect at the GABAergic synapse onto the pyramidal cell. Neurons were labelled with biocytin to confirm their morphology was pyramidal.

Although the data are convincing in general, there is one aspect of the paper that is not quite as strong: the sample size of the pyramidal cells tested in the relatively invulnerable aarea (hypothesized not to be disinhibited by MK-801) is quite small. These results are difficult to compare with the data from the much large sample from the vulnerable area. For example, 80% of cells in the vulnerable area responded, but only 47 (~57%) did so in the relatively invulnerable area. Yet 4/7 could become 80% if more cells were sampled. It could also stay at 57%; we simply don't know without more cells tested from the invulnerable area. Thus, the story would be much stronger if more cells could be sampled from the invulnerable area.

It also would be quite valuable to include the nonpyramidal cell data because those data might show directly that there was a substantial NMDA-like synaptic current that was blocked by MK-801. Of course if there was no such current it would also be important, although not supportive of the hypothesis. This issue might be addressed in part if the literature already showed that NMDA activation of interneurons is strong in the areas

that are vulnerable to MK-801; I believe there is a short paper by Jones and Buhl about this in entorhinal cortical slices. But the authors do mention that they recorded from two nonpyramidal cells; what were the results from those experiments? Even if the authors can only say that there was a long slow EPSC it would be supportive of the hypothesis.

In other aspects, the manuscript is very good. It is written extremely clearly, and the figures show high quality recordings. The length of the Introduction, Methods, etc. are appropriate. In some areas, however, there is a bit too much basic information (e.g., what is a miniature IPSC). However, that will be helpful to those who are less familiar with electrophysiology, and there may be a fair number of such readers given the broad readership of this journal. The paper should note other papers about the MK-801 induced neurodegeneration phenomenon by the Castren laboratory.

A minor point about the morphology: in the drawings, more labeling would be helpful to clarify where the layers are and the white matter. Also, please distinguish the axon from the dendrites. They are very difficult to distinguish as is because the line thickness of dendrites and axons are similar, and this makes it hard to be convinced that the cells are indeed pyramidal cells. In figure 1, borders with the white matter, borders of the general area, and layers would be useful to note.

The American Physiological Society

October 15, 2001

Please address all correspondence to:
Dr. Peter L. Strick, Editor
JOURNAL OF NEUROPHYSIOLOGY
University of Pittsburgh
W1640 Biomedical Science Tower
200 Lothrop Street
Pittsburgh, PA 15261
Phone: 412-383-9090
Fax:412-383-9061
E-mail: jnpadmin@pitt.edu
strickp@pitt.edu
Website: www.jnp.strick.pitt.edu

Dr. Swartzwelder
Neurology Research
VA Medical Center
Room 21, Building 16
508 Fulton Street
Durham NC 27705

Dear Dr. Swartzwelder:

I am happy to inform you that your revised manuscript entitled "Differential effect of ethanol on NMDA receptor-mediated EPSCs in pyramidal cells in the posterior cingulate cortex of juvenile and adult rats" (J0433-1) has been accepted for publication in the *Journal of Neurophysiology*. It has been forwarded to the office of the American Physiological Society in Bethesda, where the paper will be copyedited, sent to the printer for typesetting, and published in the next available issue. Please contact that office if you have any questions regarding the status of your article. A journal contact sheet is enclosed, so that you can reach appropriate staff at any time during the publication process.

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Thank you again for submitting this interesting work to the *Journal of Neurophysiology*.

Sincerely,

Peter L. Strick
Editor

PLS:tg
enc.